Sphingolipids in Toxoplasma gondii; synthesis and scavenging

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Abstract

Sphingolipids in *Toxoplasma gondii;* synthesis and scavenging

Christopher J. Barnes

Toxoplasma gondii is a globally endemic parasite which can cause severe illness in animal livestock and humans. Currently there are no effective drug treatments which do not have severe side effects and therefore the search for new drugs is of significant importance. Sphingolipids have been shown to be essential in many different eukaryotic cell types through their roles in cell signaling and regulatory roles in cell transport. Serine palmitoyltransferase is the first rate limiting step in biosynthesis and the inhibition of this enzyme has proved lethal in many organisms. This enzyme is currently uncharacterized in *T.gondii* and other *Apicomplexa*. In this work the evolutionary origins, function and the cellular localization of this protein were investigated. Further to this, endocytosis assays were also performed on extracellular tachyzoites to investigate the potential of the parasite to scavenge complex sphingolipids.

Sphingolipids in *Toxoplasma gondii*, synthesis and scavenging

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List of Abbreviations

AOS	α-oxoamine synthase
Ab A	Aureobasidin A
AIDS	Acquired immune deficiency
CHO	Chinese hamster ovary
Con A	Concanavalin A
DHS	Dihydrosphingosine
DRG	Dorsal root ganglia
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
His	Histidine
IPC	Phosphoinositol
KDS	3-keto-dihydrosphinganine
LB	Lysogeny broth
Lcb	Long chain base
LDL	Low density lipoprotein
ML	Maximum likelihood
mRNA	mitochondrial ribonucleic acid
NNI	Nearest Neighbour Interchanges
PBS	Phosphate Buffered Solution
PHS	Phytosphingosine
PLP	Pyridoxal 5'-phosphatase
PV	Parasitophorous vesicle
PVM	Parasitophorous vesicle membrane
rRNA	Ribosomal ribonucleic acid
STRE	Stress response element
Ura	Uracil

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"The work presented in this thesis is my own original research, except where indicated by statement or citation, and has not been submitted for any other degrees."

Acknowledgements

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Chapter 1 General Introduction

1.1.1 *Toxoplasma gondii is a widely distributed parasite*

The *Toxoplasma gondii* pathogen was discovered in hamster-like rodents the gundi (*Ctenodactylus gundii*) by Charles Nicolle at the Pasteur Institute. *Toxo*-meaning arc and *Plasma*-meaning life, is a clear indication of what Nicolle must have seen when he observed the isolated microorganism under a microscope (Nicolle and Manceaux, 1908; Janku, 1923). *T. gondii* was later isolated from the eye of a child suffering from a disseminated disease, thereby confirming humans are also a susceptible host (Sabin and Olitsky, 1937). The parasite was identified as an important zoonosis when tissue cysts isolated from livestock were shown to contain the same, so called, bradyzoite form seen in humans (Sabin, 1941).

1.2.1 The T. gondii lifecycle

Felids are the definitive hosts of *T. gondii* as was demonstrated when the highly infectious oocyst forms were discovered in the faeces of cats fed with badyzoite containing tissue cysts from infected prey (Hutchinson *et al*, 1965; **Figure 1.1.2 a.**). After ingestion the parasites invade enterocytes in the intestinal lumen to establish infection. These then differentiate into gametes to produce diploid zygotes (**c.**; Ferguson, 2002) before meiosis, oocyst formation and shedding (**d.**). In this sexual stage of the lifecycle, all progeny are clonal unless the prey is infected with multiple strains or multiple meals of infected prey are taken in quick succession. Infected cats shed for 6-8 days, producing over 100 million oocysts (Dubey and Frenkel, 1972). Post-excretion sporulation occurs leading to the formation of infectious sporozoites (**e.**). These oocysts remain stable and orally infectious for up to twelve months after excretion (Su *et al*, 2003). Oocysts are highly resistant to environmental and chemical destruction but are readily destroyed by desiccation or high temperatures (Dubey and Frenkel, 1972).

These properties mean that oocysts are able to persist and infect an intermediate host (**f.**) which can be any warm-blooded animal (including rodents, humans and livestock). Following ingestion the sporozoites excyst, invade enterocytes and differentiate to rapidly dividing tachyzoites (**g.**), these can be congenitally transmitted to foetuses (**h.**). Usually, following the brief acute tachyzoite phase, the infection becomes chronic with the formation of bradyzoite containing tissue cysts predominantly found in neural and muscle tissue (**i.**; Remington, 1974). At this stage *T. gondii* can transmitted without recourse to the definitive host by carnivorism, this is non-sexual propagation (**j.**). Alternatively, predation by a cat will precipitate the sexual cycle. It is through the ingestion of bradyzoite-contaminated raw or under-cooked meat that humans most commonly become infected (Frenkel, 1976).

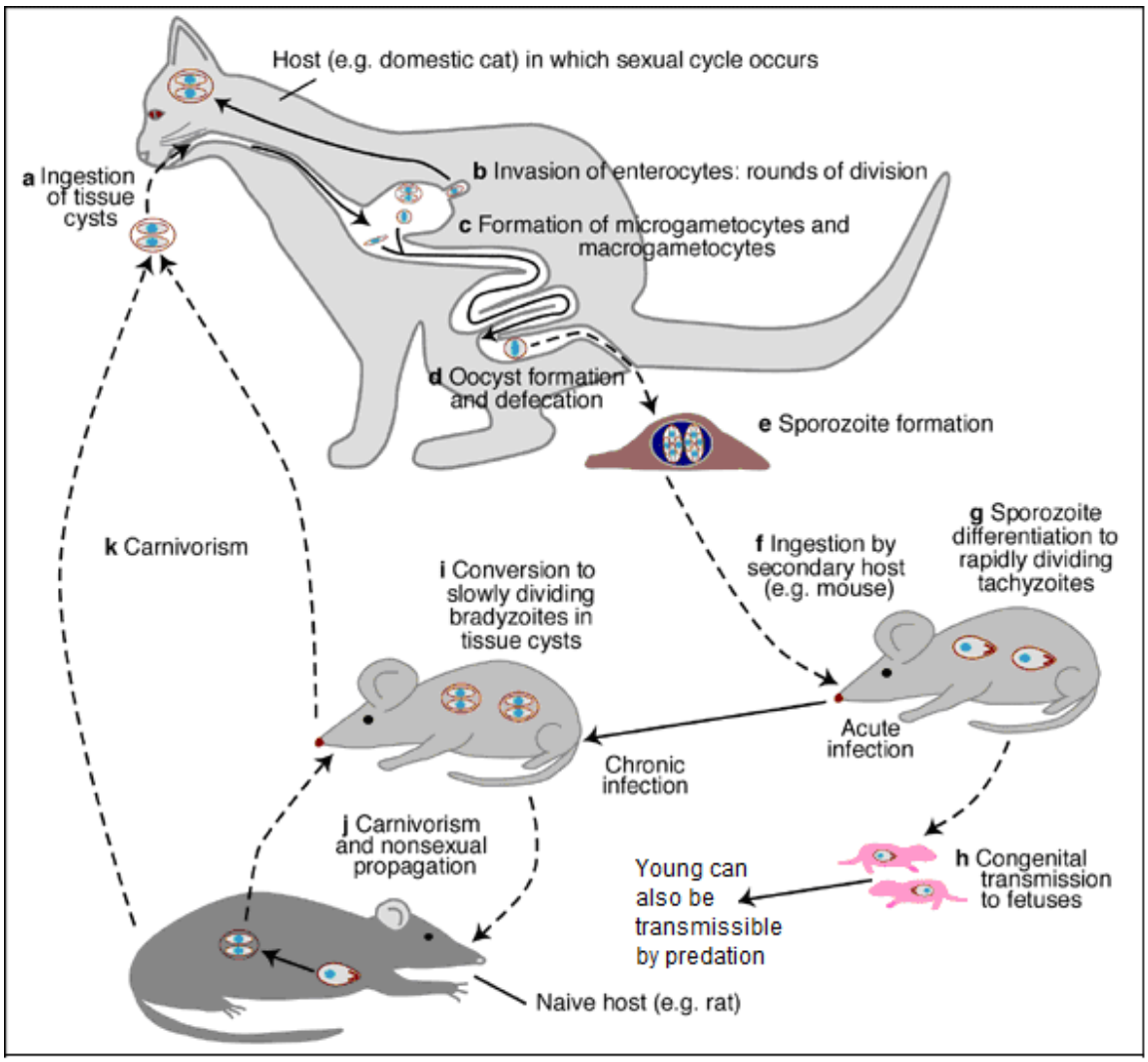

Figure 1.1.2a Life cycle of *T.gondii.* The felid species predating on infected rodents then shed infective oocysts. These can contaminate rodent food sources leading to infection and repetition of the cycle. If an infected rodent is eaten by other species the parasite can propagate non-sexually (adapted from Ajioka *et al*, 2001).

1.1.3 The interchanging life stages of T. gondii is key to its success

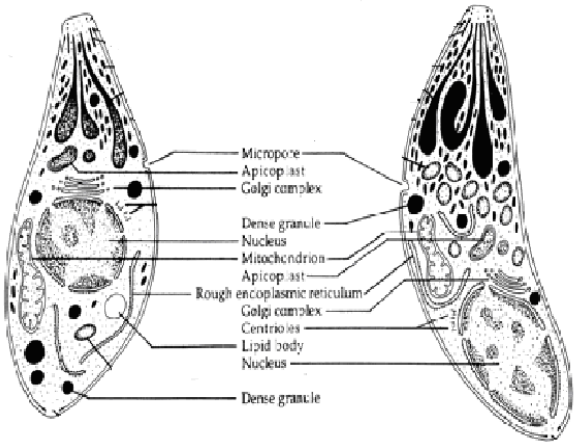

Figure 1.1.3a Schematic drawings of a tachyzoite (left) and bradyzoite (right) (adapted from Dubey *et al*, 1998)

Tachyzoites (shown in **Figure 1.1.3a, left)** are the rapidly dividing active form of the parasite which proliferate during the asexual stage of the lifecycle. They have a centrally located nucleus and are generally found during the acute phase of infections where they stimulate a strong immune response (McLeod *et al*, 1988). Bradyzoites (shown in **Figure 1.1.3a, right)** are found during the chronic phase of infection. They are slow growing parasites that do not generally elicit a potent immune response. Bradyzoites, which can be distinguished from tachyzoites by their terminally located nucleus, are found in neural and muscle tissue cysts enclosed in a resistant wall (Dubey and Frenkel, 1976). **Figure 1.1.3b** shows that bradyzoites occasionally converting into tachyzoites and entering the blood stream allow the parasite to proliferate and spread. In this scheme tachyzoites are needed to amplify enough parasites so that adequate tissue cyst bradyzoites are formed to ensure dissemination (McLeod *et al*, 1988).

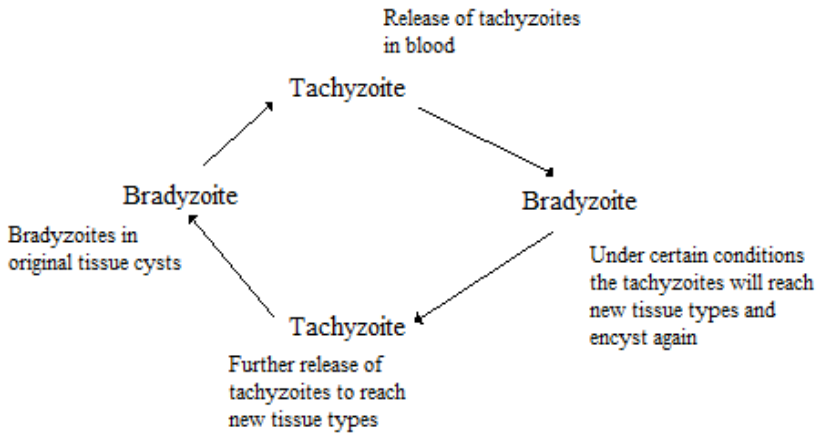

Figure 1.1.3b The transition between life-stages in the asexual lifecycle. Note how persistence is followed by expansion, which is then followed by further expansion.

Tachyzoites and bradyzoites are infectious stages formed using the asexual phase of the lifecycle. A third infectious form occurs following the complex meiotic phase of *T. gondii* replication. Oocysts are excreted with a granular sporont which completely fills them. It then contracts and two sporoblasts are apparent. The two sporoblasts then elongate to form two sporocysts which are ellipsoidal and approximately 6 – 8 μm in length. Finally four sporozoites form in the sporocysts giving rise to eight infectious forms in a single oocyst (Dubey *et al*, 1997). Sporozoites are ellipsoidal and 11 – 13μm long (shown in **Figure 1.1.3c**). Interestingly they contain a single micropyle placed randomly within the second of three cell wall layers. This is postulated to be CO^2 sensitive and to respond to bile salts and trypsin which are generally only found after ingestion in the digestive tract (Dubey *et al*, 1998; Speer *et al*, 1998).

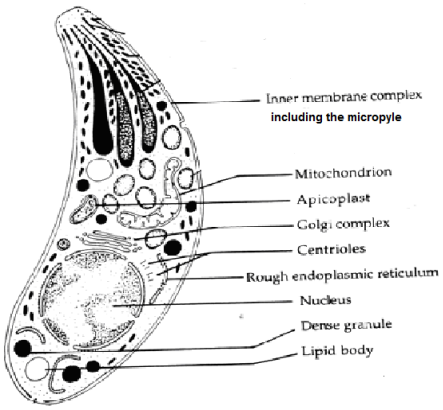

Figure 1.1.3c Schematic drawing of a sporozoite (Adapted from Dubey *et al,* 1998)

1.1.4 T.gondii is a highly successful parasite

It is the aforementioned interchange between tachyzoite and bradyzoites that makes *T. gondii* such an effective parasite, as evidenced by its global distribution (Lehmann *et al*, 2006). *T. gondii* is a haploid parasite throughout the majority of its lifecycle. However, during the sexual lifecycle in the felid definitive host, the generation of oocysts creates a diploid life-stage which undergoes meiosis. However, the progeny will be clonal if the host is infected with only one strain of *T.gondii.* Given that infection with multiple strains is a rare event and that genetic drift is uncommon, dominant strains can become prevalent and outcompete 'lesser' strains, increasing overall population fitness (Boothroyd and Griggs, 2002).

Furthermore, in contrast to other coccidian parasites, *T. gondii* does not have a strictly heterozenous (parasite has one obligatory host) lifecycles (Grigg and Sundar, 2009). This means their proliferation and spread is strictly limited to that of the host. Having a wide range of intermediate hosts along with the spore shedding definitive host is another physiological trait that allows *T .gondii* to spread far beyond the single host range (Su *et al*, 2003).

1.1.5 T. gondii as a useful genetic tool

T. gondii can be readily maintained in tissue culture and a considerable number of genetic tools available use with it. Reverse genetics in *T.gondii* is well established and genetic manipulations are now common place (Sibley and Boothroyd, 1992; Su *et al*, 2002). In contrast, other apicomplexans such as *Cryptosporidium* spp. and *Eimeria tenella* can be very difficult to be maintain and manipulate *in vitro*. The fact *T.gondii* can reproduce asexually also mean that genetically altered progeny are clonal which is often a highly desirable trait (Kim and Weiss, 2004).

1.1.6 T. gondii is a significant economic problem

T. gondii is a major cause of disease in animal livestock which therefore has a knock on economic impact. It has been shown that around 10% of miscarried lambs test positive for this protozoan parasite, making *T. gondii* the second largest cause of miscarriage, after *Chlamydia*, in the UK. Studies have detected very high levels of *T.gondii* within UK sheep populations but there are significantly higher levels of infection within the aborted population (42% base rate, 94% of aborted lambs, Duncanson *et al*, 2001). The estimated cost to this small UK agricultural subsection is £12 million per annum (Bennett and Jelpaar, 2003). Currently there are no cost effective preventative measures in place and therefore further research is needed in order to address this problem. A vaccine is currently available, Toxovax, but the initial cost and short period of protection make it generally an unviable option (Luis, 2009).

1.1.7 The evolutionary origin of T. gondii

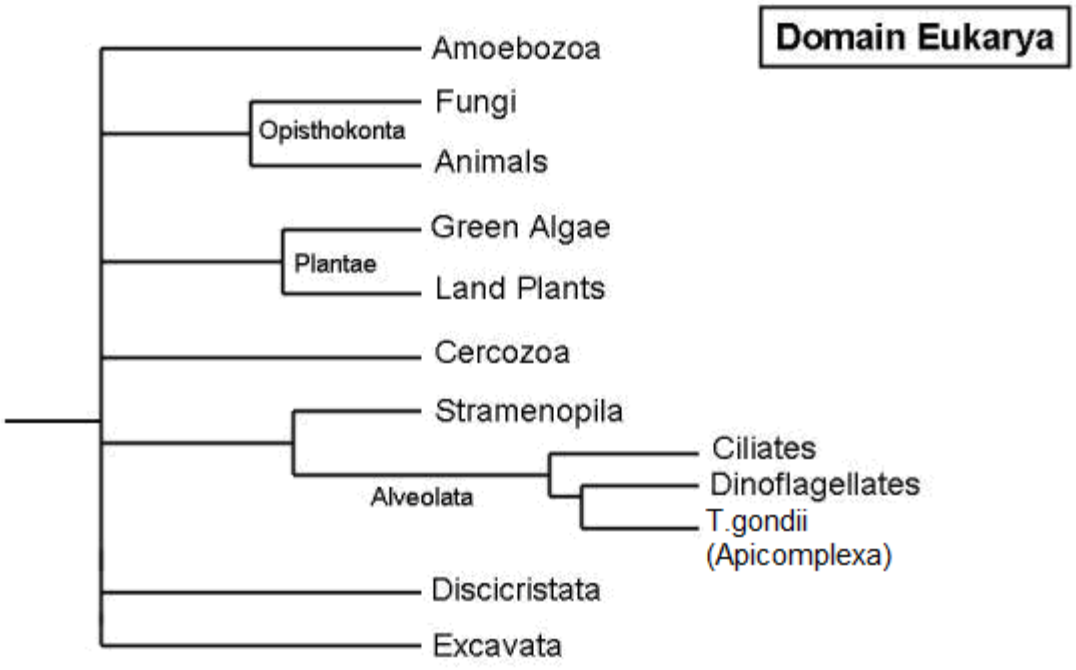

Figure 1.1.7 Evolutionary tree based on small subunit rRNA. Its shows the lineage of the Alveolata which includes *Toxoplasma* that is distinct from both Opisthokonta and Plantae from a very early stage in evolution. (adapted from Gajadhar *et al*, 1991).

The *Apicomplexa* form a clade in eukaryotic evolution containing *T .gondii* and the causative agent of cerebral malaria*, Plasmodium falciparum*. They characterized by their obligate intracellular lifecycle and they generally possess apicoplasts, an endosymbiotic organelle (Gajadhar *et al*, 1991). They have been suggested to be related to the Dinoflagellates and Ciliates, forming the Alveolata. It is highly likely that these share a common ancestor prior to speciation around 900 million years ago. Classical morphological phylogenetics and molecular (e.g. rRNA-based) modeling tend to be in agreement lending support to this hypothesis (Cavalier-Smith and Chao, 2004). **Figure 1.1.7** is a phylogenetic tree that was created using small subunit rRNA.

1.1.8 Genetic demographics

Both North America and Europe are dominated by three *T. gondii* clonal lineages. These have been dubbed clonal Types I, II, and III. The Type I genotype is always associated with lethal infection in outbred mice whilst Types II and III have been shown to be less virulent in both humans and mice (Sibley and Boothroyd, 1992). There is significantly less work done in South America and Asia but they seem to be dominated by so-called exotic strains. The atypical genotypes of the exotic strains are associated with severe infection found in immuno-competent patients. These more pathogenic strains have been shown to develop from cats that ingested several different strains simultaneously which facilitated genetic recombination (Su *et al*, 2002). As an example of this, the TgCKug2 strain isolated in Uganda was shown to be a combination of Types II and III which leads to intermediate levels of parasites (represented by cell density) in humans (Saeij *et al*, 2006).

In one notable study isolates were taken from felids in Germany which surprisingly showed only 0.25% of faecal samples were *T. gondii* positive (Hermann *et al*, 2010). The likelihood of oocyst shedding increased with the age of the cats. The majority of oocysts shed had Type II specific markers. This fits in with prior knowledge that the Type II strain dominates animal reservoirs in Europe. In the entire sample there was only one type III and no Type I specimens found. Types I and III were however more prevalent in Spain and Portugal suggesting greater diversity within Europe than first thought (Hermann *et al*, 2010). It also demonstrated there was temporal variation in oocyst shedding and therefore increased exposure risk to humans in different months of the year.

The severity of *Toxoplasma* infection varies greatly amongst individuals; a lethal case in one could be benign in another. For example Type I reproduce in two thirds of the time of Type II and III in human fibroblast foreskin cells suggesting differences in pathogenicity between strains (Boothroyd and Grigg, 2002). As an

example of how genetic diversity affects the biological traits ROP18, a key protein in host cell invasion, can be differentiated in each clonal Type. The ROP18I is found in the Type I strain which is the most invasive in both laboratory mice and humans. However, there must be ROP18I resistant hosts otherwise it would have simply out-competed the other alleles as there is no temporal or spacial separation between strains (Grigg and Sundar, 2009).

This illustrates that the shuffling of just a few epistatic genes at different loci is sufficient to produce new generations of differentially virulent progeny. A process that is aided by the incredibly high numbers of recombinant progeny (approximately 100 million) produced by a single cat. This drives the creation of phenotypes best suited for different animal hosts and explains *T. gondii* being a remarkably successful zoonosis (i.e. why it can infect so many hosts; Grigg, 2007). The recombination of different major Types seems to drive the formation of the atypical strains discussed above, which hosts have limited immunity to and hence are the most pathogenic (Grigg and Sundar, 2009).

1.1.9 Toxoplasmosis and symptoms

Infection is very common and some serious outbreaks in humans have been traced back to oocysts contaminated reservoirs (Burnett *et al*, 1998). However despite its high prevalence there are relatively few symptomatic cases. Of the symptomatic sufferers there are generally three types of infection, those that affect immuno-compromised adults, congenital infection of the newborn and severe ocular infection in immuno-competent patients. Evidence now suggests that Type II dominates AIDS and congenital infections, with 34 out of 45 *Toxoplasma* infections in France being diagnosed as Type II (Howe *et al*, 1997). Type II was also the most common congenital infection in the USA (Howe and Sibley, 1995). The Type II dominance in overt symptoms in humans is probably misleading due to its dominance within animal reservoirs.

Site	Number of Cases
Central nervous system	34
Heart	14
Lung	10
Liver	4
Kidney	4
Skeletal muscle	3
Bone marrow	3
Lymph nodes	2
Spleen	2
Other (pancreas, testis, stomach)	4

Table 1.1.9 Tissue cysts found in people who died of Toxoplasmosis (59 subjects in total; adapted from Remington, 1974)

T. gondii encysts after ingestion in many tissue types but with particularly high prevalence in the brain. The cysts have a long persistence and are responsible for lifelong infection (shown in **Table 1.1.9**; Remington, 1974). Encephalitis is caused by the reactivation of latent infections (potentially the sporadic release of tachyzoites from tissue cysts). It is still not comprehensively known whether bradyzoites have to undergo transformation to tachyzoites then back to bradyzoites to form further tissue cysts, however it is this cyst formation and subsequent reactivation that disrupts normal cell architecture and causes the most severe effects of *Toxoplasma* infection.

Ocular toxoplasmosis is a common sequela of congenital infection. This includes severe retinochorditis. When the fovea is destroyed, as is common, central vision is lost. Congenital infection can, however, lay dormant until the second or third decade of life. The most severe effects occur when a mother is infected with *Toxoplasma* for the first time in her life during the second or third trimester of

pregnancy. Here the parasite can infect the non-immune foetus causing severe damage often resulting in miscarriage. Interestingly the Type II and III have never been detected when analysing the ocular infection (Grigg and Boothroyd, 2003). As aforementioned, Type II and III dominate animal reservoirs and the development innate immunity to these but not to Type I and atypical strains is most likely responsible for enhanced pathogenicity of these strains**.** At present all such studies have been performed on relatively few subjects. However, since analysis of *Toxoplasma* DNA is part of a clinical diagnosis, more extensive genotyping studies are possible (Grigg *et al*, 2001). Larger scale studies should also include asymptomatic patients to allow greater clarify the importance of strain on pathology.

1.1.10 Immune status severely affects pathology

There is substantial morbidity and mortality caused by *T. gondii* in immuno-compromised adults and congenitally infected infants. There is also an enhanced risk of death with the greater age of the subject. In one study it was shown that no one month old Swiss-Webster Mice died after challenge with the LD50 of tissue cysts from the Me49 strain but only 25% survive the same challenge in 1 year old mice (McLeod *et al,* 1988). This is likely due to a less effective immune response in the elderly mice which is an effect likely to be replicated in humans. Effective treatment of *T. gondii* in humans is of great clinical importance for organ transplantation. Post-transplant drug-induced immune suppression greatly reduces the chance of an autoimmune response to the donor organ, but does leave patients susceptible to *T. gondii* tissue cyst reactivation, tachyzoite proliferation and spread. Previously uninfected heart donor recipients have been shown to develop life-threatening toxoplasmosis when receiving *Toxoplasma* encysted organs. This is also the case for kidney transplant patients were multiple people have died from organ allografts infected with *T. gondii* tissue cysts (Rodgers *et al*, 2008). **Table 1.1.10** shows that 6 out of 59 fatal cases arise from infected donations to previously unexposed hosts (Remington, 1974).

Underlying condition	Number of cases
Lymphoma	
Hodgkinson's disease	21
Sarcolymphoma	4
Hystiocytic lymphoma	1
Lymphogranuloma	4
Leuakemia	
Acute lymphocytic leukemia	4
Chronic lymphocytic leukemia	6
Acute myelogenous leukemia	2
Chronic myelogenous leukemia	4
Myeloid metaplasia	1
Carcinoma	
Ovary	1
Brochogenic	1
Breast	2
Neuroblastoma	1
Multiple myeloma	1
Malignant melanoma	1
Organ transplant	
Renal	4
Heart	1
Liver	1

Table 1.1.10 the underlying conditions of which Toxoplasmosis sufferers also have. The study predates the AIDS epidemic which became one of the leading causes of infection (adapted from Remington, 1974).

This study is obviously highly skewed since the AIDS epidemic, which in many areas of the globe accounts for the vast majority of toxoplasmosis cases (Zangerie *et al*, 1991). Since the advent of HIV, persons of an immuno-compromised condition have become more common place. One cohort study in America showed that of the 13 people who were suffering from AIDS and tested positively for *Toxoplasma* antibodies, 5 (38%) showed signs of toxoplasmic encephalitis. 11% of the overall population tested positive for *T. gondii* antibodies of which none of the non-AIDS sufferers showed signs of encephalitis (Isrealski *et al*, 1994). The HIV-1 virus has became a leading cause of symptomatic toxoplasmosis, although this is a retreat since the advent of anti-retrovirals.

1.2.1 Introduction to sphingolipids

Sphingolipids, which comprise a sphingoid base with an amide-linked fatty acid, are major membrane constituents that are also involved in many eukaryotic cellular processes (Kopitz, 1997). Their biosynthesis is known to be vital for the growth of yeast and mammalian cells (Pinto *et al*, 1992; Buede *et al*, 1991). Ceramide is an unmodified sphingolipid that is the base of more complex sphingolipid formation. It serves as a secondary messenger in many signalling cascades in yeast. In complex sphingolipids ceramide is modified by a polar head group such as phosphorycholine for sphingomyelin, or by carbohydrates such as galactose or glucose for glycosphingolipids (Kopitz, 1997).

1.2.2 The sphingolipid biosynthetic pathway is conserved up to formation of sphingosine production in all eukaryotic organisms

The first step of sphingolipid biosynthesis occurs in the ER and is the condensation of palmitoyl-coenzyme A with *L*-serine. This is performed by serine palmitoyltransferase enzymes which produces 3-keto-dihydrosphinganine (KDS) which is then reduced to *erythro*-sphinganine (dihydrosphingosine, DHS). These steps are ubiquitous in all known eukaryotic organisms. Subsequently, there is then divergence in evolution. In mammals ceramide is formed from DHS via ceramide synthase (Birchwood *et al*, 2001). In mammals, sphingomyelin synthase catalyses the addition of phosphorycholine from phosphatidylcholine to ceramide which forms sphingomyelin, the major complex sphingolipid. The addition of glucose or galactose moieties to ceramide makes glycosphingolipids which can be further decorated with other carbohydrate groups and sometimes sulphates to form hundreds of complex sphingolipid species (Merrill *et al*, 1996). Ceramide and complex sphingolipid are pivotal in intracellular transport and signal transduction. It is therefore important that regulation of both products and reactants is stringent (Simons and Ikonen, 1997; Muniz and Riezman, 2000).

Unlike mammals, plants and fungi produce phytoceramide from phytosphingosine via phytoceramide synthase. IPC synthase is used in both groups to produce inositol phosphorylceramide (IPC) from phosphatidylinositol and phytoceramide. IPC is subsequently further decorated to form more complex sphingolipids via, for example, mannosylation (Reggiori and Conzelmann, 1998). Mammals do not form IPC however there is evidence for this sphingolipid species in *T.gondii* (Sonda *et al*, 2005).

These differences in sphingolipid biosynthesis between mammals and fungi, make the enzymes involved in complex sphingolipid formation in pathogenic fungi ideal therapeutic targets (**Figure 1.2.2**). IPC synthase is encoded by the AUR1 gene in *S. cerevisiae*. The cyclin depsipeptide aureobasidin A (Ab A) is a fungal inhibitor of this IPC synthase which is also seemingly effective against *T. gondii* (Sonda and Hehl, 2006). Ceramide levels increase in yeast treated with Ab A as the substrate is no longer processed by IPC synthase. Ab A toxicity is a likely result of increased activation of apoptosis pathways due to ceramide acting as a secondary messenger in this process (Azzouz *et al*, 2002).

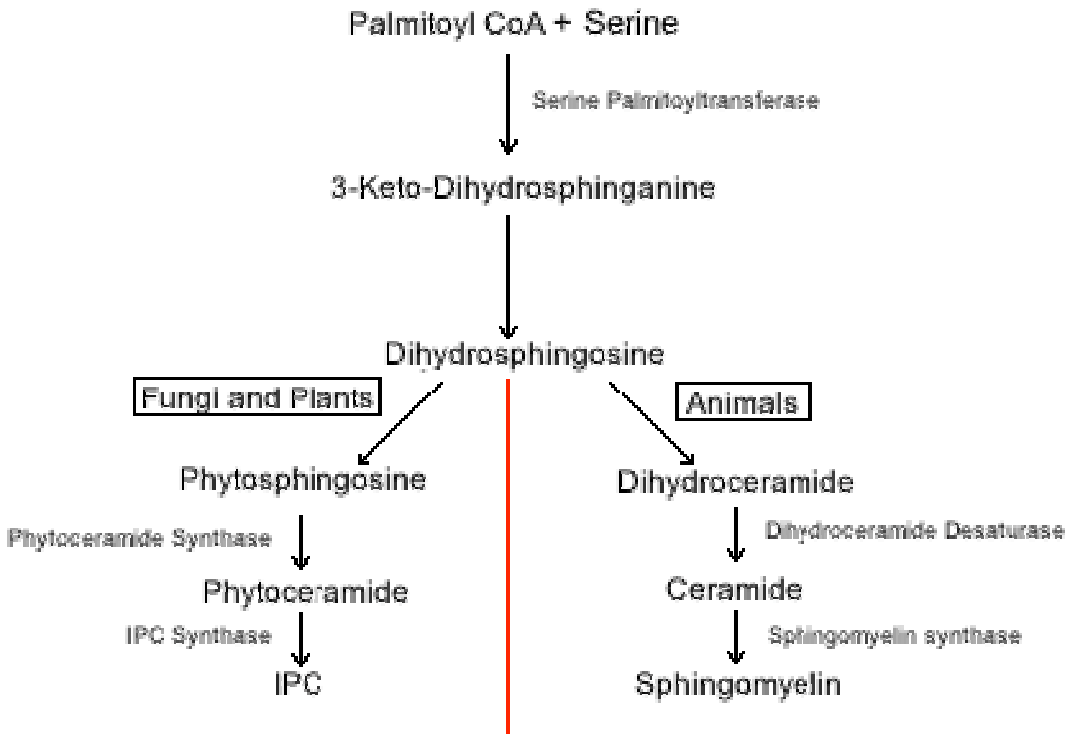

Figure 1.2.2 Schematic diagram of ceramide production. Note that Sphingomyelin Synthase activity is primarily associated with the Opithiskonta, Plantae and Fungi species can also synthesis sphingomyelin but predominantly go through the IPC pathway.

1.2.3 Sphingolipids have a range of functions in eukaryotes

Sphingolipids make up to 30% of the total phospholipids and 7% of the mass of the plasma membrane in *Saccharomyces cerevisiae* and this pattern is reflected throughout eukaryotes *(*Patton and Lester, 1991*)*. They are contained within the ER, golgi, lysosome and endosomes in membranes where they play structural and regulatory roles (Hechtberger *et al*, 1994). The degradation of sphingomyelin in the outer plasma membrane to form the secondary messenger, ceramide, occurs in response to receptors being activated (Kim *et al*, 1991). The 'activators' of the signalling pathway include things such as; cytokines, UV, ionizing radiation, heat, DNA damage and chemotherapeutic agents (Riboni *et* al, 1991; Kolesnick and Kronke, 1998; Hannun and Luberto, 2000). After stimulation, several major events can happen in cells which activate pathways involved in apoptosis, proliferation and differentiation (Riboni *et al*, 1997). Extracellular

signalling cascades can also be activated affecting multiple tissue types by stimulating immune responses or altering cell-cell interactions (Hakomori, 1996; Ballou *et al*, 1996).

1.2.4 Sphingolipids play a role in stress responses

An example of the importance sphingolipids play in cellular signalling is their role in cell stress responses. There is a two or three fold increase in dihydrosphingosine and phytosphingosine production in *S. cerevisiae* cells taken from 25˚C to 37˚C. This is linked to trehalose accumulation (Van Dijck *et al*, 1995). These lipid species can also activate stress response elements (STRE's) altering the transcription of epigenetic genes causing large scale alterations in cell physiology (Jenkins and Hannun, 2000). It is evident that the regulation of sphingolipids has global implications for a cell.

1.2.5 Sphingolipids enable essential cellular processes such as endocytosis

By controlling protein phosphorylation sphingolipids are necessary for endocytosis (Friant *et al*, 2000). Endocytosis can be restored in yeast sphingolipid mutants by introducing exogenous protein kinase activity. The inhibition of phosphatases can also be used to restore endocytic activity (Robinson *et al*, 1993). Through controlling phosphorylation sphingolipids are involved in the regulation of actin polarization and an active cortical actin cytoskeleton is necessary for endocytosis (Robinson *et al*, 1993). Similarly, sphingolipids have been shown to be important in exocytosis. The use of temperature sensitive yeast for serine palmitoyltransferase has demonstrated that sphingolipids have a role in regulating the fusion of secretary vesicles to the plasma membrane. The null phenotype could be rescued by culturing the mutants in media that contains phytosphingosine (PHS), sphingosine and ceramide (Marash and Gerst, 2001). Whilst knowledge of the regulation of sphingolipid biosynthesis and the interaction of these lipid species with other

cellular processes has advanced throughout the years, the molecular mechanisms underlying this are still not fully understood and this will be a key area of future research (Dickson, 1998; Dickinson and Lester, 1999).

1.3.1 Serine palmitoyltransferase was originally isolated in Saccaromyces cerevisiae

In nearly all eukaryotes the serine palmitoyltransferase function resides as a heterodimeric enzyme complex consisting of the Lcb1 and Lcb2 subunits (Hanada *et al*, 1998). The two subunits are synthesised from individual genes and dimerize at a 1:1 ratio after posttranscriptional modification. The two genes, LCB1 and LCB2, were originally isolated in *Saccharomyces cerevisiae* by analysis of complemented mutant strains defective in SPT activity (Buede *et al*, 1991; Nagiec *et al*, 1994). Lcb1 null mutant yeast are absolutely auxotrophic for sphingosine and only become viable and proliferate when phytosphingosine is added to the growth medium. Lcb1 and 2 orthologues have been isolated from many other eukaryotes including mice and humans (Nagiec *et al*, 1996; Hanada *et al*, 1999; Weiss and Stoffel, 1997). The mouse Lcb1 and human Lcb1 proteins are about 30% identical and 36% similar at the amino acid level (Hanada *et al*, 1999).

1.3.2 There is a colocalization of Lcb1 and Lcb2 proteins with increased SPT activity

Screening of functional mutants in *S. cerevisiae* has shown that mutations in either LCB1 or 2 causes a lack of SPT activity (Buede *et al*, 1991). Further evidence to suggest an association between the Lcb1:Lcb2 complex and SPT activity comes from analysis of mRNA expression in different tissue types. High levels of Lcb1 and Lcb2 mRNA are found in the brain, lungs and kidney, which also corresponded with high SPT activity in rats whilst both were particularly low in the testis (Hanada *et al*, 1999).

The high sequence homology with pyrodoxal-5'-phosphate (PLP)-dependant enzymes led to the assumption that Lcb2 contained a PLP binding site. This motif is absent in Lcb1. Lcb2 carries a lysine residue which is expected to form a

Schiff base with PLP and therefore Lcb1 and Lcb2 have been speculated to be regulatory and catalytic units respectively (Nagiec, 1996; Hanada *et al*, 1998; Mukherjee and Dekker, 1990).

There is however conflicting evidence over the role of Lcb1. It was originally thought through sequence functional analyses, that the mouse and Chinese Hamster Ovary orthologues of the yeast Lcb1 protein were essential for SPT activity (Buede *et al*, 1991; Pinto *et al*, 1991). Conversely to this, other research showed that over expression of mouse LCB2 mRNA by four times wild type levels in HEK (human embryonic kidney) cells was sufficient to raise serine palmitoyltransferase activity by three-fold (Weiss and Stoffel, 1997). This could potentially be due to an excess of Lcb1 which is then 'paired' with the additional Lcb2. However, this contradicts work in yeast where only when levels of both Lcb1 and Lcb2 were raised was there an increase in SPT activity (Buede *et al*, 1991). Although the functional role of Lcb1 in mammals is still not fully understood it is worth noting that all other studies in other eukaryotes have shown both Lcb1 and Lcb2 to be necessary for SPT activity (Pinto *et al*; 1992; Hanada *et al* 1998; Buede *et al*, 1991).

1.3.3 There is tight regulation of Lcb2

There are differences between the mRNA levels of Lcb1 and Lcb2 (Weiss and Stoffel, 1997). The relatively low level of Lcb2 protein compared to mRNA could be explained by co-translational down-regulation or post-translational degradation. It is however known that the regulatory process must be stringent as there is no detectable free sphinganine or increase in ceramde even when exogenous SPT activity introduced. `It is therefore likely that sphingoid bases are converted to ceramides which are immediately shuttled into further biosynthesis pathways. Tight regulation is indicative of the severe negative effects on host cells if there are fluctuations in levels of either product or reactant (ceramide is a signalling molecule for apoptosis). This hypothesis is supported by work

indicating that sphingosine is a potent protein kinase C inhibitor which would be hugely debilitating to a cell (Hannun *et al*, 1989). Interestingly there are similarities in phenotypes of sphingolipid starved yeast and starved inositol auxotrophs, as both suffer from loss of lipid synthesis and hence could be indicative of loss-of-function mutations rather than cytotoxic effects (Culbertson and Henry, 1975). In either hypothesis however it is evident both the production and correct abundance of sphingolipid intermediates is essential for correct cellular function.

1.3.4 Neuropathy conditions occur in humans with Lcb1 mutations

It has long been known that mutations in the yeast sphingolipid biosynthetic pathway can severely damage cell viability. Defects in human gene orthologues have now been shown to cause disease. The HSN1 gene (NIM 162400) on chromosome 9q22 encodes for Lcb1 in humans. It is particularly highly expressed in dorsal root ganglia (DRG) and it is this gene that is mutated in hereditary neuropathy conditions (Bejaoui *et al*, 2001). Orthologous HSN1 yeast mutants show a retained ability to form the Lcb1:Lcb2 heterodimer, but that the HSN1 mutations in Lcb1 were centered around the catalytic lysine in Lcb2 that forms the PLP-Schiff base motif assumed to be responsible for maintaining the three dimensional active site (Gable *et al*, 2002). Mutations in conserved LCB2 catalytic regions can also cause HSN1-type phenotypes (Auer-Grumbach, 2008).

Hereditary sensory neuropathy type 1 (HSN1) is a slowly progressive neurological condition in which the most obvious symptom is distal sensory loss and autonomic disturbances. Other symptoms include variable muscle wasting and chronic skin ulcers. In more severe cases, complications are spontaneous fractures, osteomyelitus and necrosis. Neurotic anthropathy is also a symptom which could lead to amputations (Dyck *et al*, 1993).

1.4.1 Bacterial SPT function is encoded by a single gene

Most bacterial species do not produce sphingolipids. However, a water-soluble SPT has been identified in *Bacteroides melaninogenicus*, a commensal of the upper respiratory tract, although purification of the activity was not achieved (Lev and Milford, 1981). Most notably, alphaproteobacteria sphingomonads have been shown to possess SPT activity via ^{14}C-serine labelling of formed 3-keto-dihydrosphinganine (KDS). *Sphingomonas paucimobilis* has a significantly higher level of SPT activity than many other sphingomonads and hence it is the species in which lipid composition and structure have been most extensively investigated (Yabuchi *et al*, 1979; Yano *et al*, 1982). It was demonstrated that *S. paucimobilis* SPT could be over-expressed in *E. coli*, refuting the previous theory that the difficulty in expressing enzyme orthologues was due to toxic accumulation of KDS. On isolating the enzyme from *E. coli* it was discovered that SPT function is performed by a water-soluble homodimer (Ikushiro *et al*, 2001), in complete contrast to the eukaryotic heterodimer which is a membrane-bound ER resident enzyme (Mukherjee and Dekker, 1990; Mandon *et al*, 1992**)**.

1.4.2 Conserved amino acids in bacterial SPTs are the key to catalytic activity

The structure of the *S. paucimobilis* SPT was determined by X-ray crystallography and key amino acid residues in the active site identified (Yard *et al*, 2007). A lysine residue, Lys-267, forms a Schiff base linkage with the PLP co-factor in a manner typical of amino acid transferases, including both eukaryotic and prokaryotic SPT. However, there are other catalytically important residues which interact directly with PLP and are conserved within other SPTs (Yard *et al*, 2007). For example, His-159 stacks above one face of the pyridinium ring of PLP and is important for catalytic function. It is located at the beginning of a three-residue motif that is strictly conserved in the α-oxoamine synthase (AOS) family to which SPT belongs (Yard *et al*, 2007).

1.4.3 The SPT homoodimer is conserved amongst other sphingomonads

The bacterium *Sphingobacterium multivorum* SPT has since been SPT crystallized. It demonstrated strong homology to the *S. paucimobilus* enzyme and complete conservation of the catalytic domain (Ikushiro *et al*, 2009). This strongly supported that notion that the bacterial SPT is an intimately interlocked homodimer (Yard *et al*, 2007, Ikushiro *et al*, 2009).

1.4.4 There are many similarities between Lcb1, Lcb2 and bacterial SPT proteins

Lcb2 and bacterial SPT proteins are classified as members of the PLP-dependent OAS family (Nagiec *et al*, 1994). In addition, there is sequence homology at the active site with other amino acid synthases such as 5-aminolevulinic acid synthase (ALA-synthase) and 2-amino-3-ketobutyrate CoA ligase (the catalytic subunit of the complex). Furthermore, the *E. coli* 2-amino-3-ketobutyrate CoA ligase uses pyridoxal phosphate as a cofactor, like Lcb2 and SPT indicating a conserved mechanism of action (Buede *et al*, 1991).

Analyses of bacterial SPT and eukaryotic Lcb1 and 2 amino acid sequences indicated, with a high degree of certainty, the two have a shared ancestry. However, it is likely that the proto-SPT underwent gene duplication event early in eukaryotic evolution (Ikushiro *et al*, 2001), leading to the almost ubiquitous ER membrane bound heterodimeric enzyme complex (Mukherjee and Dekker, 1990; Mandon *et al*, 1992). Evidently this event did not occur in the sphingomonad bacteria which maintain homodimeric, soluble SPT (Ikushiro *et al*, 2003; Ikushiro *et al*, 2007).

Naturally with this deep-rooted evolutionary divergence there are some differences between the prokaryotic and eukaryotic enzymes. For example the bacterial SPTs are less strict in binding to different acyl-CoA substrates. In addition, in the eukaryotes evolution of the Lcb1:Lcb2 heterodimer and the

proposed regulation of function could have conferred the role for the enzyme in both intra and extracellular sphingolipid signalling (Ikushiro *et al*, 2001). However, what is clear is that the conserved active site residues give the specificity of SPT function in both bacteria and eukaryotes (Ikushiro *et al*, 2009).

1.4.5 There is evidence for novel eukaryotic SPTs

Sphingolipids and *de novo* SPT activity have been described in *T. gondii* by analyses of parasites labelled with tritiated serine (a substrate of SPT; Azzouz *et al*, 2002). The gene(s) encoding SPT remained unidentified in the genome sequences of *T. gondii* and others apicomplexans. However, recently putative SPTs have been identified in most of the *Apicomplexa*, including *T. gondii* (Denny *et al*, unpublished). They show significant divergence from both the eukaryotic and bacterial SPTs, although in most species are encoded by a single gene as in the prokaryotes. As such the origin of this putative, apicomplexan enzyme remains unclear.

1.5.1 The role of sphingolipids in T. gondii for survival and proliferation

The invasive tachyzoite cells of *T. gondii* require sphingolipid for the formation of the parasitophorous vesicle (PV; Coppens *et al*, 2000). This increases the demand for sphingolipid by tachyzoites which will require rapid sphingolipid biosynthetic or scavenging pathways (Mordue, 1999). It is yet to be determined whether the *T. gondii* parasite is able to utilise sphingolipids from the host cell without initial endogenous sphingolipid production. However, it has been established that these lipids are scavenged from the host once the PVM is formed (de Melo *et al*, 1991; Mordue, 1999). However, host sphingolipid synthesis has only a minor role in *T. gondii* proliferation (Pratt and Denny, unpublished).

1.5.2 Sphingolipids and cholesterol are essential for GPI-anchored protein trafficking

Inhibition of ceramide synthesis in yeast inhibits the transport of GPI-anchored proteins (Bagnat *et al*, 2000). This maybe due to the role of sphingolipids, along with cholesterol, in the formation of membrane microdomains (lipid 'rafts') which are thought to be important in the intracellular trafficking of GPI-anchored proteins (Futerman and Hannun, 2004; Dickson, 1998).

Lipid rafts may be thought to have a similar role in the transport of GPI-anchored molecules in *T. gondii*. However, notably *T. gondii* are auxotrophic for cholesterol and parasites have been shown to scavenge this lipid, along with others, from the host cell (Coppens *et al*, 2000, Charron and Sibley, 2002). Without this scavenging PV formation is impaired and given the association of cholesterol and sphingolipids in rafts, it is likely that both these species are essential for parasite survival and proliferation.

1.5.3 Cholesterol uptake is via an abnormal endocytic pathway

In normal endocytic transport, molecules are excluded from the surrounding area of the *T. gondii* PVM (Mordue *et al* 1999). However, it is predicted that LDL-containing endosomes and lysosomes are hydrolyzed into free cholesterol at the PV (Coppens *et al*, 2000). In support of this chloroquine, which impairs lysosomal function, causes an accumulation of LDL and a decrease in cholesterol reaching the PV which inhibits parasite proliferation. It is hypothesised that *T. gondii* may either actively divert host sterol-carrier protein or synthesise its own which transports cholesterol to the PVM (Coppens *et al*, 2000). The scavenging of cholesterol may prove to be linked to that of sphingolipid. Sphingomyelin has been demonstrated to be rapidly sequestered by intracellular *T. gondii* (Pratt and Denny, unpublished). However, as discussed above *T. gondii* synthesizes these sphingolipids *de novo* and the role of this and host synthesis in proliferation remains unclear.

1.5.4 Targeting T. gondii sphingolipid biosynthesis with drugs

Many current treatments for toxoplasmosis are characterized by unpleasant side effects meaning that new drugs and targets are needed (Mordue *et al*, 1999). Sphingolipid biosynthesis has long been a target for novel anti-fungals, particularly the non-mammalian IPC synthase (Georgopapadakou, 2000). As discussed it has been suggested that *T. gondii* possess an IPC synthase and, furthermore, that this activity is susceptible to the ‘gold standard’ fungal inhibitor, auereobasidin A (Ab A; Sonda et al, 2005). Verification of the role of *de novo T. gondii* sphingolipid synthesis in parasite proliferation, coupled with the identification of divergent key enzymes (SPT and IPC synthase), may lead to the identification of new drug targets and also inhibitors.

1.6 Conclusions

Toxoplasma gondii remains a widely distributed parasite of huge economic and health importance. It causes miscarriages in animal livestock, as well as untold deaths in areas where the HIV is uncontrolled. It is also a potential risk to the unborn throughout the developed and developing worlds, and can cause miscarriage or very severe post-natal symptoms, even in the second or third decade life. These include life-threatening encephalitis and ocular infection leading to the loss of central vision.

Research into sphingolipid biosynthesis and function is a growing area of interest in many organisms, but work on *T. gondii* and other apicomplexans is limited. Given the identification of key but divergent enzymes, together with the evidence for both scavenging and *de novo* synthesis of sphingolipids, further research may elucidate novel drug targets and the nature of the interaction between the host and the *T. gondii* pathogen.

1.7 Aims and Objectives

1. Investigation of the origin of the divergent apicomplexan SPT using a phylogenetic approach.

2. Functional analyses of *T. gondii* TgSPT1 using an auxotrophic yeast Lcb2 mutant line. Is it a functional SPT?

3. Subcellular localisation of TgSPT1. Is it ER localized like other eukaryotic SPTs?

4. Analyses of *T. gondii* endocytosis. Can the parasites take up sphingomyelin, dextran or concanavalin A?

Chapter 2 Materials and Methods

2.1 Utilized mutant yeast strains

The ScLcb2 deficient yeast strain was a kind gift from Hossam Shams-Eldin, University of Marburg. The strain, YPH499–HIS–GAL–LCB2 *S. cerevisiae* was constructed in YPH499 [Mat a; ura3-52; lys2-801amber; ade2-101ochre; trp1-63; his3-200; leu2-1] (Stratagene) as described previously (Shams-Eldin *et al*., 2005). An engineered GAL1/HIS3-cassette was transformed into the YPH499 strain and transformants selected on minimal media lacking histidine but containing galactose. Replacement of the ScLCB2 promotor with the GAL promotor fragment was confirmed by PCR.

The AGD61-27 mutant was a kind gift from Prof Robert Dickinson (University of Kentucky; Nagiec *et al*, 1997). This strain (MATα *ura3-52 leu2-3, 112 lcb1::URA3 SLC1-1 lys2-80*^{1amber} *ade1 ipc1-1 ade1*) lacks LCB1, and so lacks serine palmitoyltransferase activity. It also has a mutation within the IPC synthase gene. This is the *SLC1-1* (sphingolipid compensatory) mutation, which means it can grow by synthesis of novel glycerophospholipids which at least partially compensate for the loss of the sphingolipids. The loss of Lcb1 allowed mutation of IPC synthase without the accumulation of the toxic intermediates of sphingolipid biosynthesis (such as 3-keto-dihydroshphingosine). AGD27-67 colonies can then be identified by their unusually large ellipsoidal morphology.

2.2 Instruments and Equipment

Make	Model	Function
Becker-Coulter	Avanti J-20XP	Centrifuge
Boeco	320	Centrifuge
Sigma	Jan-14	Microcentrifuge
Techne	TC312	Thermocycler
Olympus	1x71	Fluorescence Microscope
Retiga	1300	Attached microscope camera
Boeco	S-30	Spectrophotometer
Biotek	FLx800	Fluorescence plate reader
BioRad	Universal HoodII	Agarose and YPD imaging

Table 2.2.1 List of equipment used.

2.3 Primers, Buffers and Media Compositions

2.3.1 *Primers*

Primer Name	Sequence
TgSPT1MfeIF (Full length)	GGCAATTGATGTTCGGAAGCGTCTTTGTC
TgSPT1NsiIR	GGATGCATGCAGGGATATAGGTCCGTCTTCC
TgSPTEcoRI (1-49)	CCGGAATTCCGTCGTTGAAAAAAGCCAGGAG
TgSPT1 SpeI for pRS426	ACTAGTATGGCTTCGGGTGCAACGTACTTC
TgSPT1 HindIII for pRS426	CGCAAGCTTTCATCGGAGCATGTCAGTGGGTGGG
ScLcb2 EcoRI for pRS426	GGGGAATTCATGAGTACTCCTGCAAACTATACCCG
ScLCcb2 XhoI fpr pRS426	GGGCTCGAGTTAATTAACAAAATACTTGTCGTCCTTACAATC

Table 2.3.1 List of Primers used.

2.3.2 Buffers and Media Compositions

Solution / Media	Vol. Weight	Stock Solution	Company
YPD	10g/l 20g/l 20/l Ad. Water	Bactoyeast extract Bactopeptone Dextrose	Melford Melford Sigma
SGR-Plates	44.4g/l 22.2g/l 22.2g/l Ad. Water	Galactose Raffinose Agarose	Sigma Sigma Sigma
SD-Plates	22.2g/l 22.2g/l Ad. Water	Glucose Agar	Sigma BDH electran
Amino acids mix-his-ura	17.0g/l 50.0g/l 40mg/ml 60mg/ml 60mg/ml 40mg/ml Ad. Water	Yeast Nitrogen Base NH_4SO_4 Adenine Leucine Lysine Tryptophane	Melford Melford Sigma Sigma Sigma Sigma
LB agar	35.6g/l Ad. Water	LB agar Ezmix powder	Sigma
LB broth	20g/l Ad. Water	LB Broth	Sigma

Dulbecco's Modified Eagle Medium	17.25g/l 3.7g/l Ad. Water	 DMEM powder Sodium bicarbonate	 Gibco Sigma
DMEM serum-free with BSA	17.25g/l 3.7g/l 50g/l Ad. Water	 DMEM powder Sodium bicarbonate Fat-free Bovine serum albumin	 Gibvo Sigma Sigma
DMEM w/supplements	17.25g/l 3.7g/l 100ml/l 0.1mM 5µg/l Ad. Water	 DMEM powder Sodium bicarbonate FCS Non-essentail amino acids Penicillin Streptomycin	 Gibco Sigma BioSera Gibco Gibco
Cytomix	120mM/l 0.15mM/l 10mM/l 25mM/l 2mM/l 5mM/l 1.2g/l 15.2g/l Ad. Water	Sodium chloride Calcium chloride Dipotassium hydrogen phosphate HEPES pH7.6 EGTA Magnesium chloride ATP glutathione	Sigma Sigma Sigma Sigma Sigma Sigma Sigma Sigma
Phosphate Buffered Solution	5 tables/l Ad. Water	PBS tablets	Sigma

Table 2.3.2 List of buffers and media used.

2.4 Standard techniques and protocols

2.4.1 Bioinformatics

2.4.1 Step I Sequence sampling strategy

To ensure a broad range of sampling and to maintain taxon cardinality whilst avoiding the effects of paralogy, protein sequences were taken from the four major eukaryote supergroups. Lcb1, Lcb2 and SPT orthologues were isolated by BLAST searches (Altschul *et al*, 1997). The initial databases searched were from the National Centre of Biotechnology (NCBI) (www.ncbi.nlm.nih.gov) and the Joint Genome Institute (http://genome.jgi-psf.org/). The apicomplexan and *amoebozoan* sequences were identified by BLAST searches with the putative *Toxoplasma gondii* SPT in GeneDB (www.genedb.org). The accession numbers and sources of sequences can be found in **8.1**.

2.4.1 Step II Removal of non-homologous regions and long-branch effects

The sequences were edited after ClustalW alignments (http://www.ebi.ac.uk/Tools/clustalw2/index.html; Thompson *et al*, 1994). Only regions which showed unambiguous conservation were kept for further analysis. Initial quick, non-bootstrapped neighbour-joining phylogenetic trees were constructed using the Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/) and sequences which showed long-branch effects were deleted. This facilitated a more statistically robust analysis. As a result of this sequences from; *Arabidopsis thaliana, Oryza sativa*, *Ostreococcus tauri*, *Cyanidioschyzon merolae*, *Giardia intestinalis*, *Cyanidioschyzon merolae*, *Phytophora ramorum, Tetrahymena thermophila, Emiliania huxleyi, Eh050 virus,* and *Oryza sativa* were removed. It is worth mentioning that a homologous protein of the fellow

apicomplexans *Theileria annulata* and *Theileria parva* could not be found, however there databases are not complete.

2.4.1 Step III Phylogenetic analyses

A final ClustalW alignment was performed without the deleted long branching sequences. Following hand editing of the alignment phylogenetic trees were inferred using protein maximum likelihood analyses. The PhyML (Phylogenetic Most Likelihood) was performed using the Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/). This programme uses a fast algorithm and Nearest Neighbour Interchanges (NNI) to generate phylogenetic trees (Guindon and Gascuel, 2003). An alternative analysis was performed using the RaxML (Randomized Axelerated Maximum Likelihood) on the Phylobench bioinformatics platform (http://phylobench.vital-it.ch/raxml-bb; Stamatakis *et al*, 2008). Using this package a distance model (P) is computed via a Jones-Taylor-Thornton matrix and a neighbor-joining tree constructed from this (Jones *et al*, 1992). In both cases bootstrap values were calculated to assess the robustness of tree nodes.

2.4.2 TgSPT1 functional assays

2.4.2 Step I pRS426-ScLcb2 and pRS426-TgSPT1 plasmid construction

The TgSPT1 gene was amplified by PCR using *Pfu* (Promega) and primers TgSPT1 SpeI and TgSPT1 HindIII (listed in **Table 2.3.1**) from cloned cDNA (Pratt and Denny) according to manufacturers protocols. Following isolation using a QIAquick PCR Purification Kit (Qiagen) the PCR product was digested with SpeI and HindIII (Promega) and ligated using T4 DNA ligase according to manufacturers protocol (Promega), into the yeast expression vector pRS426 (**Figure 2.4.2**) to create pRS426-TgSPT1. pRS426-ScLCB2 was created in much the same way as pRS426-TgSPT1. In this case, PCR amplification of ScLCB2 was from *S. cerevisiae* genomic DNA (Sigma) using primers ScLCB2 EcoRI and ScLCB2 XhoI listed in **Table 2.3.1**.

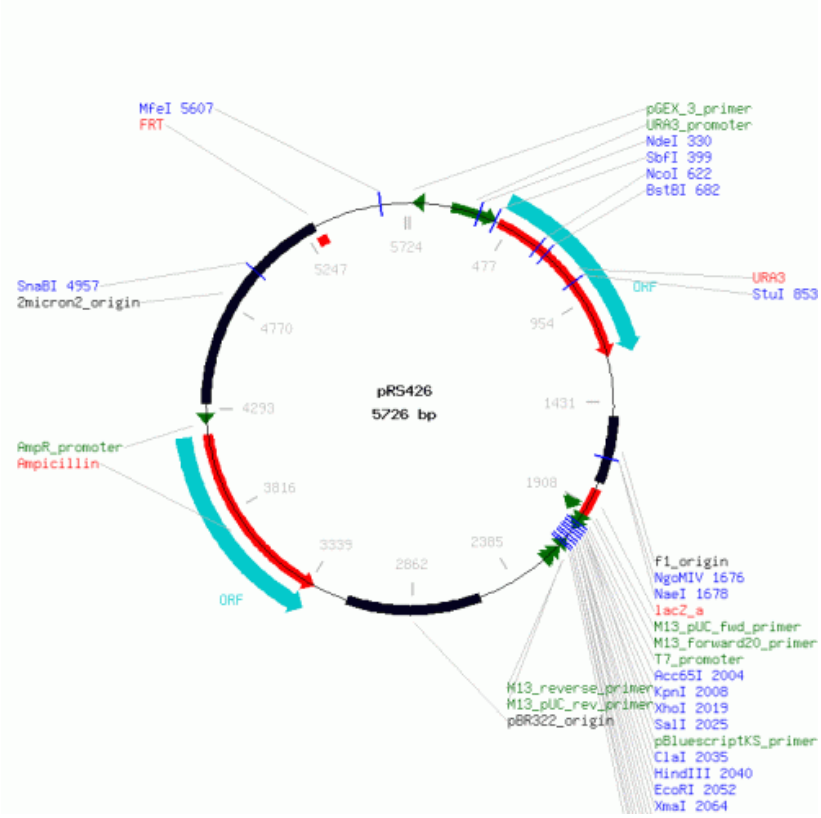

Figure 2.4.2 ScLCB2 and TgSPT1 were cloned into the yeast expression vector pRS426.

In each case clones were selected on LB ampicillin, after transformation into MachT1 DH5α *E.coli* (Invitrogen) competent cells, which was performed as per manufacturer instructions. Subsequently, plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAgen). Clones were confirmed by appropriate restriction digestion (Promega) and fractionation on a 1% agarose gel with 5 µg/ml ethidium bromide. A 1kb DNA ladder was employed (Promega).

2.4.2 Step II Complementation Assays

The transformation of pRS426-ScLCB2 and pRS426-TgSPT1 into the ScLCB2 conditional mutant yeast strain (YPH499–HIS–GAL–LCB2) was performed as per the MATCHMAKER GAL4 Two-hybrid System 3 users manual and complemented mutants selected on $SGR^{-His-Ura}$ plates (competency is achieved via lithium acetate exposure followed by heatshock). The growth of the YPH499–

HIS–GAL–LCB2 transformed with pRS426-ScLcb2, pRS426-TgSPT1 and empty pRS426 was compared on permissive $SGR^{-His-Ura}$ and non-permissive $SD^{-His-Ura}$ plates. After 5 days at 30°C the plates were visualized using the EPI-white configuration on a BIO-RAD Universal Hood II and the Quantity One 4.5.0 basic software package.

2.4.2 Step III Diffusion Assays

All three yeast strains (the AGD-67 strain and the two transformed lines) were grown to exponential phase (OD_{600} 0.6) at 30°C in either YPD (AGD) or $SD^{-His-Ura}$ liquid. 1 OD unit of each cell line was resuspended in YPD with 0.8% agarose at 42°C, poured into plates and allowed to set. Subsequently, Myriocin (1 mM in DMSO, Sigma), Aureobasidin A (25 μM in DMSO, Clontech) and DMSO (Sigma) were spotted onto the plate in 1 µl, 2 µl and 3 µl volumes. The plates were then incubated at 30°C for 3 days and the results were analysed using a BIO-RAD Universal Hood II as above.

2.4.3 Localization Studies

2.4.3 Step I pT8-TgSPT1-Ty and pT8-TgSPT1-49-GFP-Ty plasmid construction

The pT8-Myc-GFP-Ty plasmid (pT8mycGFPPfmyoAtailTy-HX) is a custom-made expression vector for *T. gondii*, provided courtesy of Prof Dominique Soldati of the University of Geneva . The cloning of TgSPT1 and TgSPT1-49 into the vector was originally performed by Dr. Steven Pratt of the University of Durham. The full length open reading frame was amplified from cDNA using *Pfu* DNA polymerase (Promega) and the primers TgSPT1MfeIF (Full length) and TgSPT1NsiIR (listed in **2.3.1**). The product was then digested with NsiI (Fermentas) and MfeI (Promega). pT8-GFP-Myc plasmid was digested with EcoRI (Promega) before *Pfu* was used to create a blunt end. NsiI digestion was then performed before alkaline phosphatase treatment (CIAP, Promega). After purification of both cut

plasmid and PCR product, which were purified using the QIAquick Gel Extraction Kit (QIAgen), ligation was undertaken and selected as above.

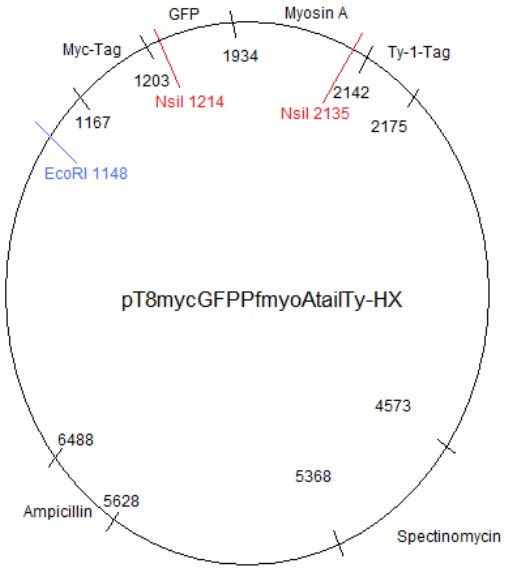

Figure 2.4.3 Schematic drawing of the pT8-GFP-Myc *T. gondii* expression vector. The NsiI and EcoRI restriction sites were used in order to clone in the TgSPT1 and TgSPT1-49 PCR fragments.

The TgSPTEcoRI (1-49) and TgSPT1NsiIR primers listed in **Table 2.3.1** were used to amplify the SPT leader sequence as before. The product was digested first with MfeI and blunt ended as above, then with EcoRI. After purification the product was ligated into pT8-Myc-GFP-Ty, cut with EcoRI and treated with CIAP, and clones selected as before. pT8-TgSPT1-49-GFP-Ty clones were then verified by PCR.

Plasmids were isolated using a QIAgen Midiprep Kit according to manufacturers protocol. Removal of the final 70% ethanol wash was carried out in a Category II microbiology hood to ensure sterility. At least 50µg of plasmid was then resuspended in 100µl of cytomix.

2.4.3 Step II Cell Culture and Preparation

Vero (kidney epithelial cells from African Green Monkey), used as host cells, were maintained in DMEM with supplements (**2.3.2**) at 37°C, 5% CO_2. *Toxoplasma gondii* strain RHΔHX was maintained within these Vero cells.

For transfections, Vero cells were trypsinized (0.025%, Gibco), counted using a Neubauer haemocytometer and 10^5 of cells were transferred to each well of a 24-well plates (Nunc) containing 0.9cm round microscope cover slips (Sarstedt). This was then incubated as above for 16-18 hours.

RHΔHX *T. gondii* were isolated from infected Vero cells when there was lysis of the monolayer. Parasites were prepared for transfections by ensuring host cells lysis by serial passage through a 25G hyperdermic syringe (Terumo), before filtering through 5µm and then 3 µm polycarbonate filters (Millipore). Isolated parasites were pelleted by centrifuging at low speed to improve cell viability (2,500g, Boeco U-320R) for 5 minutes and resuspending in cytomix at $1x10^7$/ml.

2.4.3 Step III Transfection

The 100µl of plasmid DNA suspension was combined with 300µl of the isolated parasites in cytomix and the transient transfection protocol was followed exactly as in Weiss and Kim (2007). Briefly, the mixture was added to a 2mm electroporation cuvette (Cell Projects) before electroporation (1.5 kV pulse, 25Ω resistance and 25µF capacitance) using a BTX 630 cell manipulator (Harvard Instruments). Parasites were then transferred directly into the prepared Vero cells within a 24-well plate and incubated for 24-36 hours.

2.4.3 Step IV Fluorescence microscopy of transiently transfected Toxoplasma

Transfected parasites were fixed by the addition of 10% formaldehyde and after 15 minutes at room temperature the cells were washed with phosphate buffered solution (PBS) five times. After incubation in PBS, 5% FCS and 0.1% Triton X-100 was added for 30 minutes. Cells were then labeled for 1 hour at room temperature with the αTy monoclonal antibody (a kind gift of Prof Keith Gull, University of Oxford) at 1:100 in PBS, 5% FCS and 0.1% Triton X-100. After washing 3 times in PBS, the secondary antibody was applied, Alexafluor 594 goat anti-mouse (Invitrogen) 1:200 in PBS, 5% FCS and 0.1% Triton X-100. After a further 1 hour incubation and a PBS wash, the cells were incubated with DAPI (4,6-diamidino-2-phenylindole, 300nM) at room temperature for 15 minutes. Finally, the cover slip was removed from the 24-well plate, mounted on a microscope slide and images captured using an epi-fluorescence microscope (Olympus 1x71) and a CCD (Retiga 1300). The resulting images were manipulated using the OpenLab 4.0.1 software package.

2.4.4 Endocytosis Assays

2.4.4 Step I Cell Preparation

T. gondii RHΔHX tachyzoite parasites were isolated from Vero cells as above (2.4.3 step II). Isolated parasites were pelleted at 2,500g (Boeco U-320R) and resuspended at 10^7/ml in serum free DMEM and incubated at 37°C for 30 minutes.

2.4.4 Step IIa Dextran Endocytosis Assay

500µl of parasites was placed in two separate 1.5ml eppendorf tubes; one of which was preincubated on ice for a further 30 minutes, the other at 37°C in a water bath (Grant JB series). At this point, 10µl of FITC-Dextran (10 mg/ml, 10

kDa, Invitrogen Molecular Probes) was added to each sample. The tubes were then incubated for another two hours at their respective temperatures before fixation as above (2.4.3 step IV) for fluorescence microscopy and quantitation using a fluorescence plate reader as described in *Step III*.

2.4.4 Step IIb Concanavalin A Endocytosis Assay

200 µl of parasites were resuspended in ice-cold serum free DMEM ($1x10^7$/ml) and incubation for 30 minutes in ice. 2 µl of Concanavalin A Texas Red conjugate (Invitrogen; 5 mg/ml lyophilized) was then added to the sample, before a further 30 minutes on ice, followed by incubation at 37°C for 30 minutes. Following fixation, samples were processed for fluorescence microscopy as above (2.4.3 step IV) or quantitation using a fluorescence plate reader as described in *Step III*.

2.4.4 Step IIc BODIPY-Sphingomyelin Endocytosis Assay

Four 500µl aliquots of parasites were pelleted and the media replaced with ice-cold serum-free DMEM ($1x10^7$/ml). After incubation on ice for 30 minutes, 5 µl/ 1mg/ml in DMSO of BODIPY-sphingomyelin (Invitrogen Molecular Probes) was added to each sample prior to a further 30 minute incubation on ice. The samples were pelleted and resuspended in the warm (37°C) serum-free media for the 0, 5, 15 and 60 minute periods respectively. After the allotted time period, samples were pelleted, washed 3 times in ice-cold serum free DMEM and left on ice for the duration of the other experiments. After 60 minutes the samples were washed again and resuspended in 500µl serum-free DMEM 5% fat-free bovine serum albumin (Sigma). These were then incubated on ice for 30 minutes to back-extract fluorescent sphingomyelin from the *T. gondii* plasma membrane (Pagano *et al*, 1991). Following fixation, samples were processed for fluorescence microscopy as above (2.4.3 step IV) or quantitation using a fluorescence plate reader as described in *Step III*

2.4.4 Step III Processing samples for fluorescence plate reader

The FITC-dextran and BODIPY-sphingomyelin labelled *T. gondii* were analysed using a fluorescence plate reader (Biotek FLx800). To give reproducible results, cells were fixed, washed and resuspended in PBS at 10^7/ml (equilibrating after counting if necessary). 100µl of each sample was loaded into a 96-well optical plate (Sigma) in triplicate and 460/40 and 540/35 excitation-emission filters were used. 100µl of PBS was loaded to measure background fluorescence levels which were then subtracted from the sample readings.

Chapter 3 Bioinformatic analyses of the predicted apicomplexan serine palmitoyltransferase

3.1.1 Aims and Introduction

The aim of this part of the project was to begin to elucidate the evolutionary origin of the *Apicocomplexa* serine palmitoyltransferase (SPT). An activity known to be present in *Toxoplasma gondii* (Azzouz et al, 2002) but encoded by previously unidentified genes.

3.1.2 Introduction to SPT activity genetics

Previous data have shown that the eukaryotic SPT is a heterodimer encoded by two genes, LCB1 and LCB2 (Hanada et al, 1998). In contrast the sphingomonad bacterial SPT is active as a homodimer and is encoded by a single gene (Ikushiro *et al*, 2003). Two highly related genes encoding predicted PLP-dependent class II aminotransferases both with some homology to eukaryotic SPT subunit 2 and its prokaryotic equivalent, have been found in the *T. gondii* sequence database (www.toxodb.org; Denny, unpublished). These represent the only predicted class II aminotransferases in the database of unknown function and so by default the putative SPT. Close orthologues of the these predicted proteins are founds in other apicomplexans such as *Plasmodium falciparum* (the causative agent of malaria; www.plasmodb.org) and *Eimeria tenella* (coccidiosis in poultry; www.sanger.ac.uk/Projects/E_tenella). However, in each case only a single gene encoding the putative SPT is evident. Therefore it seems likely that the two *T. gondii* genes arose as a result of a gene duplication event and that the apicomplexan putative SPT is encoded by a single gene in most species. In this it resembles the sphingomonad bacteria (Ikushiro *et al*, 2003).

In light of these observations, it is of great interest to investigate the evolutionary origin of the putative apicomplexan SPT which, in primary sequence, diverges

considerably from the eukaryotic heterodimeric enzyme. This was achieved in a phylogenetic study using distance analyses.

3.1.3 Sequences analysed

The following is a list of the sequences used in the analyses along with their labels used for short. They were aligned using ClustalW and then, after editing by hand, phylogenetic trees inferred by maximum likelihood using RaxML and PhyML. A full list with accession numbers and species excluded is included in **Appendix 8.1.**

Label	Organism of Origin
Opisthokonta	
Metazoa	
HsLcb1	*Homo sapiens*
HsLlcb2	*Homo sapiens*
MmLcb1	*Mus musculus*
MmLcb2	*Mus musculus*
DmLcb1	*Drosophila melanogaster*
DmLcb2	*Drosophila melanogaster*
CeLcb1	*Caenorhabditis elegans*
CeLcb2	*Caenorhabditis elegans*
DrLcb1	*Danio rerio*
DrLcb2	*Danio rerio*
NvLcb1	*Nematostella vectensis*
NvLcb2	*Nematostella vectensis*
MbLcb1	*Monosiga brevicollis*
MbLcb2	*Monosiga brevicollis*
Fungi	
ScLcb1	*Saccharomyces cerevisiae*
ScLcb2	*Saccharomyces cerevisiae*
CnLcb1	*Cryptococcus neoformans*
CnLcb2	*Cryptococcus neoformans*
SpLcb1	*Schizosaccharomyces pombe*
SpLcb2	*Schizosaccharomyces pombe*
AnLcb1	*Aspergillus nidulans*
AnLcb2	*Aspergillus nidulans*
RoLcb1	*Rhizopus oryzae*
RoLcb2	*Rhizopus oryzae*
Archaeplastida	
PtLcb1	*Populus trichocarpa*
PtLCB2	*Populus trichocarpa*
Chromalveolata	
Apicomplexa	
TgSPT1	*Toxoplasma gondii*
TgSPT2	*Toxoplasma gondii*
PfSPT	*Plasmodium falciparum*
PvSPT	*Plasmodium vivax*
EtSPT	*Eimeria tenella*
CmSPT	*Cryptosporidium muris*
Excavata	
Discicristata	
TbLcb1	*Trypanosoma brucei*
TbLcb2	*Trypanosoma brucei*
LmLcb1	*Leishmania major*
LmLcb2	*Leishmania major*

Sphingomonads

SpSPT *Sphingomonas paucimobilis*
SmSPT *Sphingobacterium multivorum*

3.2.1 Analysis of SPT and Lcb2 sequence alignment

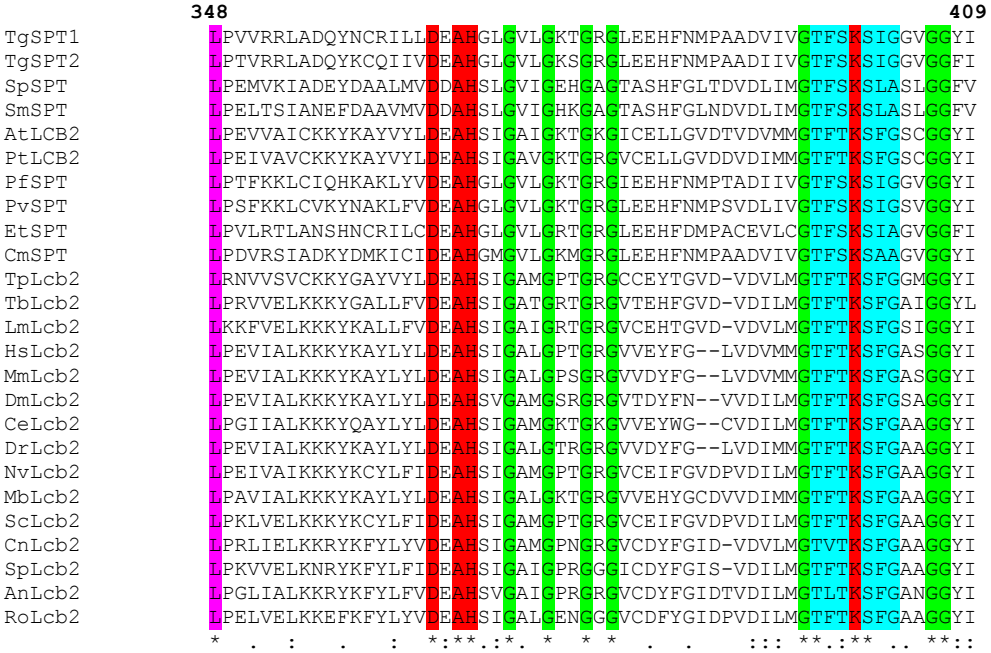

Figure 3.2.1a ClustalW alignment of SPT/Lcb2 active site domain from across evolution. The alignment of a region equivalent to amino acids 348-409 of TgSPT1. The red DXAH and lysine residues and 6 blue residues highlighted have previously been described in *Sphingomonas paucimobilis* as important for catalytic function (Ikushiro *et al*, 2003). Green highlighted residues are completely conserved glycine residues which have not been previously characterised. Pink indicates a single conserved leucine residue also not previously described.

Figure 3.2.1a is a sequence alignment comprising of the putative active site domain of SPT and Lcb2 proteins (ClustalW2; Thompson *et al*, 1994; Felsenstein, 1989). The red DXAH and lysine residues, along with the three residues either side of the lysine (blue) are highlighted as they have previously been suggested as being important for catalytic function (Ikushiro *et al*, 2003). Of these residues, the key lysine is conserved in every sequence, which emphasises its importance. X-ray crystallography has shown that this residue forms the Schiff base-PLP-binding loop which is essential for serine palmitoyltransferase activity (Yard *et al*, 2007). It is therefore unsurprising that it is extremely rigidly maintained as mutations at this residue would be hugely

debilitating to enzyme function and ultimately to the organism. The six residues highlighted (blue) either side of this lysine have also been predicted to be of importance and 3 of the 6 amino acids are relatively well maintained compared to the majority of the sequence. They have been suggested to be of importance in presenting the lysine residue in such a way that allows the lysine residue to form an external Schiff base with *L*-serine (Ikushiro *et al*, 2009). The potential structural importance of these residues is reflected in conserved amino acids (33.3% similarity, 16.7% of residues showing no conservation). It should be noted that the threonine and serine residues within this domain are ubiquitously conserved in every species.

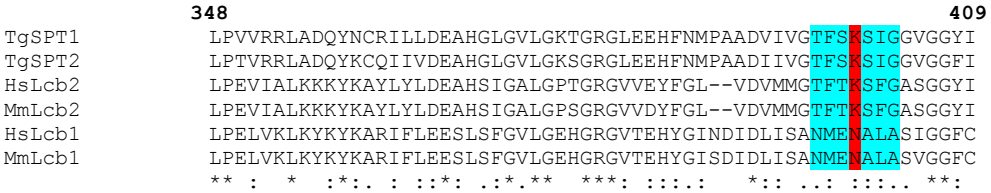

Figure 3.2.1b ClustalW sequence alignment of Toxoplasma SPTs, HsLCB1 and 2 (*Homo sapiens)* and MmLCB1 and 2 (*Mus musculus)*. This is a region equivalent to amino acids 348-409 of TgSPT1. The catalytically essential lysine residue is not conserved in the LCB1 sequences.

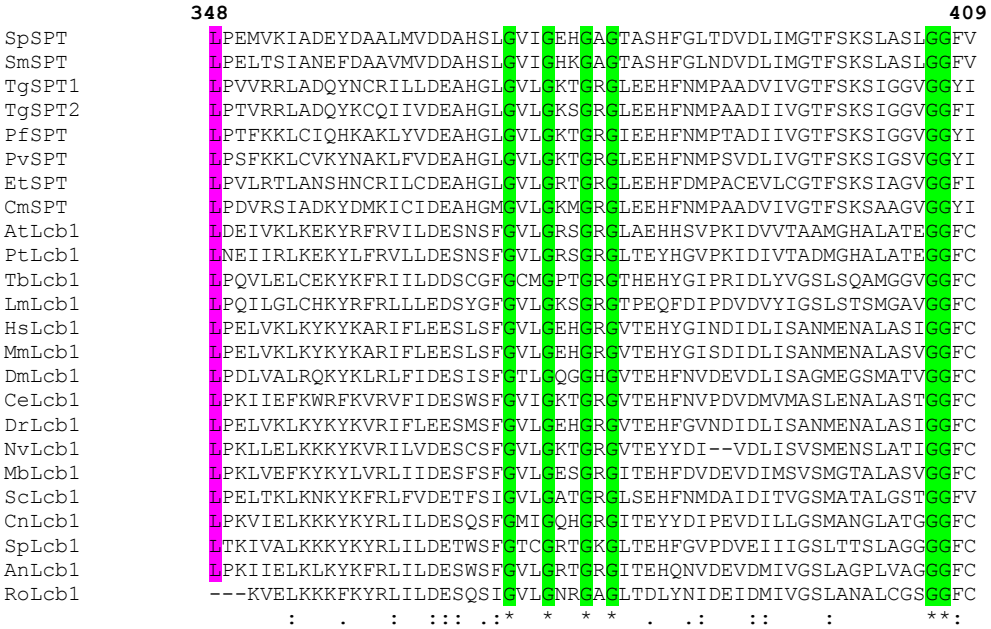

Figure 3.2.1c ClustalW sequence alignment of bacterial SPTs, apicomlexan SPTs and eukaryotic Lcb1s. This is a region equivalent to amino acids 348-409 sequence of TgSPT1. Note the conserved glycine residues (green) found within all the predicted proteins, including the eukaryotic Lcb1 which is thought to have a regulatory rather than a catalytic role.

It is also worth noting that the lysine residue, and indeed the 7 amino acid binding motif (the red and blue highlighted region of **Figure 3.2.1b**), are not conserved in Lcb1 sequences (Mukherjee and Dekker, 1990; Hanada *et al*, 1998). This reflects the fact that the catalytically active domain is solely within the Lcb2 subunit, with the Lcb1 thought to have a regulatory role. Given the level of homology observed between the Lcb1 and Lcb2 predicted proteins it is thought the heterodimeric structure of the enzyme is the result of a gene duplication event. This evolutionary event may have freed Lcb1 to mutate at a higher rate at residues constrained in Lcb2 due to their involvement in catalysis (Hanada *et al*, 1998). **Figure 3.2.1c** shows there are areas of conservation within the Lcb1 group itself, such as the universally conserved glycine residues (green). This suggests they have areas of specific structural importance and these could be required to enact their proposed regulatory role in the eukaryote heterodimer (Mukherjee and Dekker, 1990).

In structural terms glysine is the smallest amino acid and rotates freely adding flexibility to the protein chain. It can therefore act as a pivot in coiled structures (Doolittle, 1989). There is also a universally conserved leucine residue (pink) within this region. Leucines have strong helical coil formation potential and are one of the most abundant amino acids within inner helical cores. The presence of this leucine, coupled with the glycine residues, suggests a coiled structure which is perhaps essential for enzyme activity (Chou and Fasman, 1974). Mutating these residues by site-directed mutagenesis in the soluble, easy to assay prokaryotic SPT would allow investigation of whether they have a strong influence on the function and structure of the protein.

3.3.1 Preliminary phylogenetic analysis

Initially a maximum likelihood neighbor-joining tree (RaxML) was constructed using 40 different sequences: 8 bacterial and apicomplexan SPTs and 16 Lcb1 proteins with their Lcb2 partners. The resulting preliminary tree separated weakly

into four distinct groups; the Lcb1s, Lcb2s, bacterial SPTs and *Apicomplexa* SPTs (data not shown). These groupings were consistent with previous studies showing that Lcb1 and Lcb2 formed distinct clades but appeared to share a common ancestor (Dacks and Dolittle, 2001). They also support the distinctive nature of the prokaryotic SPTs (Ikushiro *et al*, 2003).

To investigate the evolutionary relationship between the conventional eukaryotic, prokaryotic and apicomplexan proteins, those sequences which showed long branch effects were discarded and the alignments reanalysed in the absence of either Lcb1 or Lcb2.

3.4.1 Phylogenetic analyses of bacterial SPT, apicomplexan *SPT and LCB1 sequences*

A bootstrapped neighbor-joining tree was constructed using RaxML (Nearest Neighbor Interchanges matrix) and PhyML (Jones-Taylor-Thornton matrix) maximum likelihood searches. **Figure 3.4.1** is the resultant tree which clearly demonstrates that the Lcb1, bacterial SPT and the *Apicomplexa* proteins form distinct clades. Both algorithms support this with the bacterial proteins forming a group (100%/100% RaxML/PhyML) separate from the *Apicomplexa* SPTs (100%/81%) and the eukaryotic Lcb1 sequences (99%/100%). The apicomplexan clade does tree with the eukaryotic Lcb1 group, although the level of support is equivocal (47%/82%). This provides tentative support for the apicomplexan SPT being more closely related to the eukaryotic Lcb1 than its bacterial functional orthologues.

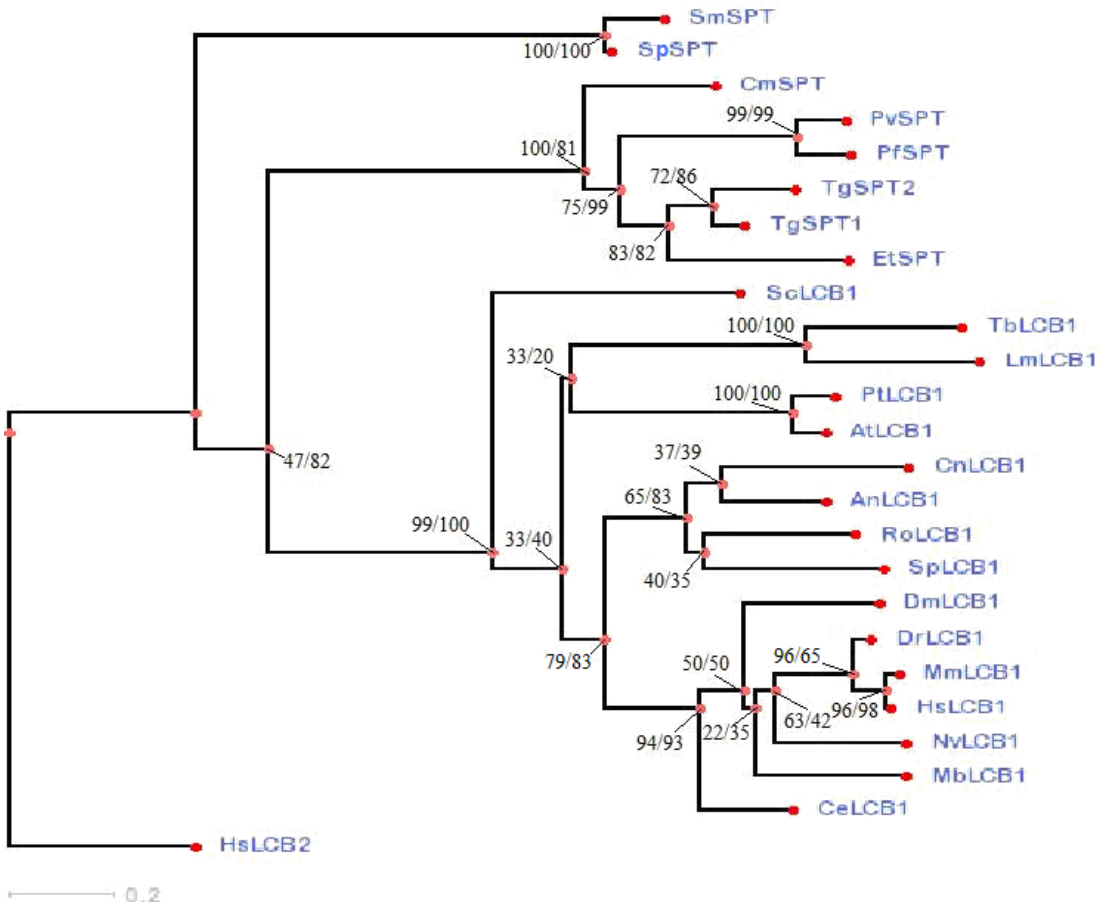

Figure 3.4.1 Phylogenetic analysis of prokaryotic SPT, apicomplexan SPT and eukaryotic Lcb1. HsLcb2 is the out group in this analysis. Values shown are RaxML/PhyML bootstrap values The prokaryotic, apicomplexan and other eukaryotic sequences clearly from 3 distinct clades, although the apicomplexan SPTs demonstrate a tentative association with the Lcb1 sequences (47%/82% bootstrap support).

3.4.2 Phylogenetic analyses of prokaryotic SPT, apicomplexa SPT and LCB2 sequences

Unlike the *Apicomplexa* and bacterial SPTs and the eukaryotic Lcb2 subunit, Lcb1 does not encode the catalytic domain (**Figure 3.2.1b**). Therefore, in order to improve the statistical confidence of the analysis (as expressed by high bootstrap values) the Lcb1 sequences were excluded and Lcb2 used in their place, thereby comparing only catalytically active SPT enzymes/subunits.

The key finding of this analysis **(Figure 3.4.2)** is that the *Apicomplexa* and bacterial SPT peptides clearly form a clade distinct from the eukaryotic Lcb2

group. The relationship of the apicomplexan and prokaryotic sequences is given some support by bootstrap values (81%/49% RaxML/PhyML). The Lcb2 clade is supported by values of 67% and 60% (RaxML and PhyML). This provides support for the apicomplexan SPT having a bacterial origin distinct from that of the other eukaryotes. This is further supported by the fact that both bacterial and apicomplexan SPTs are encoded by a single gene and therefore do not function in a heterodimer like Lcb1 and 2 in all other eukaryotes. *T. gondii* is in exception to this, encoding TgSPT1 and TgSPT2. However, unlike Lcb1 and 2 the parasite's predicted protein share a significant level of homology (approximately 80%) and both maintain the active site residues. This indicates that they do not represent two subunits of an Lcb heterodimer, and it is likely that TgSPT1 and TgSPT2 are a result of a gene duplication event that, somehow, conferred a selective advantage over the wild type. It could be postulated that such an event is the first step towards the formation of a heterodimeric SPT.

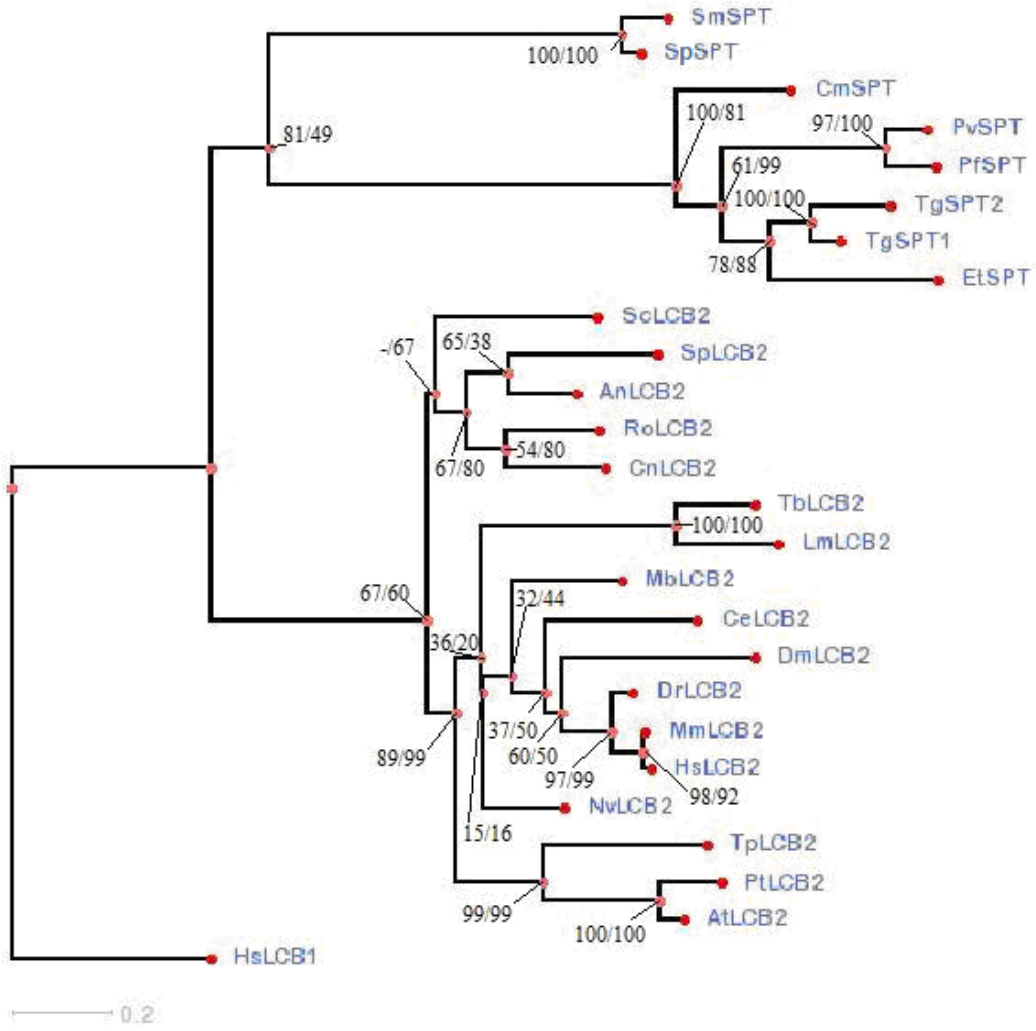

Figure 3.4.2 Phylogenetic analysis of the apicomplexan SPTs with bacterial SPTs and eukaryotic Lcb2 proteins. Values shown are RaxML/PhyML bootstrap values. The *Apicomplexa* and bacterial sequences form a group distinct from the Lcb2 clade. This indicates that they may share an evolutionary origin.

3.5.1 Future work based on bioinformatics of sphingolipid biosynthesis in the apicomplexa

Further phylogenetic analyses of other enzymes within the apicomplexan sphingolipid biosynthetic pathway would show if a general evolutionary pattern is observed. It was not until sphingomonad species such as *Sphingomonas paucimobilis* and *Sphingobacterium multivorum* were discovered that there was any convincing evidence of the capacity of bacteria to synthesize sphingolipids (Ikushiro *et al*, 2001; Ikushiro *et al*, 2007). It has been demonstrated however

they do not have homologous enzymes of the eukaryotic pathways from ceramide synthase onwards (Minamino *et al*, 2003). *T. gondii* has been demonstrated to possess enzymes for the synthesis of complex sphingolipid species, for example the IPC synthase (Sonda and Hehl, 2006; Wansadhipathi-Kannangara and Denny, unpublished). Therefore, in *T. gondii* at least, apicomplexan sphingolipid biosynthesis appears to represent a chimera of prokaroyotic-like (SPT) and conventional eukaryotic (IPC synthase) enzymes. It could be hypothesized that the SPT was obtained by lateral transfer from a bacterial species, displacing an existing Lcb1/2 enzyme, whilst the other eukaryotic enzymes in the pathway were retained. Further analyses of these other enzymes, as they become characterised, could provide support for this. Diffusion assays in **Chapter 4** and cellular localisation work in **Chapter 5** of the TgSPT1 enzyme will give biochemical and physiological information which would aid the classification of this novel protein.

Chapter 4 Complementation assays in *Saccaromyces cerevisiae*

4.1.1 Aims and Introductions

The initial aim was to demonstrate that *Toxoplasma gondii* TgSPT1 can complement a ScLcb2 deficient *Saccharomyces cerevisiae* strain, thus indicating it has serine palmitoyltransferase activity.

4.2.1 Introduction to diffusion assays in Saccaromyces cerevisiae

Sphingolipid biosynthesis has been proven to be essential in *S. cerevisiae, Histoplasma capsulatum* and *Candida albicans* (Lester and Dickinson, 1996; Welle *et al*, 1996*)*. Notably, all the steps in sphingolipid biosynthesis are conserved in eukaryotic cells to the formation of dihydrosphingosine (DHS). Subsequently, there is a divergence, as sphingomyelin synthase utilizes ceramide to form sphingomyelin in animal cells, whilst in the fungi and plant kingdoms phytoceramide is used in the synthesis of inositol phytoceramide (IPC) through IPC synthase (Sugimoto *et al*, 2004). Because of this divergence sphingolipid biosynthesis has long been recognised as a therapeutic target for specific anti-fungals.

Myriocin is an atypical amino acid which is a potent inhibitor of serine palmitoyltransferase (SPT), an enzyme which mediates the first step in sphingolipid biosynthesis. At 1 μM myriocin inhibited SPT function in Chinese hamster ovary (CHO) cells by approximately 95% and had a similar effect on cell growth. However, the growth phenotype was almost fully rescued by the addition of exogenous sphingosine, the product of the inhibited enzyme SPT. Similarly, the addition of sphingosine negated myriocin growth inhibition of mouse cytotoxic T cell lines (CTLL-2) (Miyake *et al*, 1995). These data, together with the observed resistance of the SPT deficient LY-B CHO strain to the drug (Hanada *et al*,

2000), indicated that myriocin is a specific inhibitor of the first enzyme in eukaryotic sphingolipid biosynthesis.

Incubation of leaves from the plant *Nicotiana benthamiana* with myriocin for 5 days had no impact on phenotype. However, levels of the pathogenic bacteria *Pseudomonas cichorii* did rise 10-fold during treatment. It is therefore thought that myriocin suppresses the anti-pathogenic response of the plant against *P.cichorii*, presumably via inhibition of SPT (Takahashi *et al*, 2009).

The *Saccharomyces cerevisiae* mutant *S .cerevisiae* AG27-61 (MATα *ura3-52 leu2-3, 112 lcb1::URA3 SLC1-1 lys2-80*^{1amber} *ade1 ipc1-1*) was developed in the Dickinson laboratory and has proven useful in the study of sphingolipid synthesis (Nagiec *et al*, 1997). The strain carries several gene mutations. Firstly, there is a deletion of the LCB1 gene needed to form a functional SPT heterodimer (Buede *et* al, 1991). Loss of this normally essential gene is compensated by a point mutation that creates the suppressor gene, *SLC1-1* (Nagiec *et al*, 1993). *SLC1-1* allows the formation of novel glycerophospholipids which at least partially compensate for the loss of the sphingolipids. This background allowed the selection of an IPC synthase (*AUR1*) mutant since the loss of SPT function stops a toxic accumulation of its product, 3-ketosphinganine. In this study the diploid version of this strain, AGD27-61 (Nagiec *et al,* 1997), has been used as a negative control. The absence of SPT making it resistant to myriocin, and the lack of IPC synthase AUR1p) making the potent inhibitor aureobasidin A ineffectual.

4.3.1 TgSPT1 complementation assay

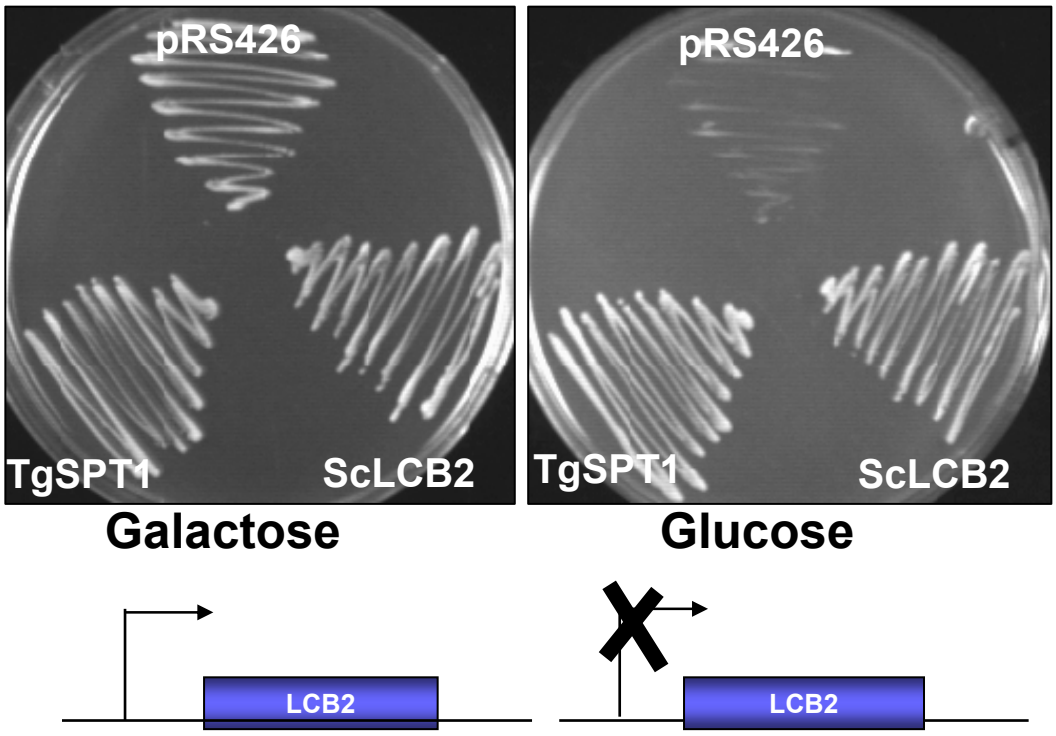

Figure 4.3.1 Transformed auxotrophic yeast grown on selective media with either galactose or glucose. Both ScLcb2 and TgSPT1 rescue the mutant *S.cerevisiae* which are deficient in endogenous ScLcb2 when grown in the presence of glucose. pRS246 is empty vector control.

Figure 4.3.1 shows the ScLcb2 auxotrophic yeast mutant described transformed with the expression vector pRS426, either empty or containing an open reading frame. In this strain the endogenous ScLcb2 gene has been placed under the control of a GAL promotor. Therefore in presence of glucose and absence of galactose the expression of ScLcb2 is suppressed and the yeast cannot grow. Unsurprisingly, when transformed with pRS426 encoding ScLcb2 the mutant Lcb auxotrophic strain is able to grow in the presence of either glucose or galactose. In contrast, transformation with empty pRS426 did not allow the yeast to grow when utilizing glucose. Like ScLcb2, expression of TgSPT1 from pRS426 was demonstrated to rescue growth under glucose conditions. This indicated that it is highly likely to represent the *T. gondii* SPT. This was confirmed in a parallel study where it was demonstrated to catalyse the production of 3-dihydrosphinganine

when expressed in *E.coli* (Pratt and Denny, unpublished). It is predicted that TgSPT1 rescues the ScLcb2 deficient mutant in one of two ways: either TgSPT1 constitutes SPT activity alone (maybe by forming homodimers); or it forms an active heterodimer with ScLcb1 in much the same way as ScLcb2. This is potentially an area for further research. By creating further auxotrophic mutants, deficient in ScLcb1 or ScLcb1 and ScLcb2, the ability of TgSPT1 to complement these could be analysed in order to unpick the role of the protozoan protein in the observed complementation (**figure 4.3.1**).

4.4.1 Drug diffusion assays

Figure 4.4.1 is EPI white images of the various yeast strains described above embedded into YPD agarose as described and having 1-3 μl of the SPT inhibitor myriocin (1 mM in DMSO), aureobasidin A (25 μM in DMSO) or DMSO diffused into the solid media before incubation at 30 °C. In all cases the negative control (DMSO) shows no zone of growth exclusion. In contrast, the specific fungal IPC synthase inhibitor aureobasidin A (Ab A) clearly prevents growth, at all volumes applied, of the ScLcb2 auxotroph complemented with either ScLcb2 or TgSPT1. However, whilst the ScLcb2 strain is clearly sensitive to myriocin confirming SPT as essential for growth (Van Middleworth *et al*, 1992), TgSPT1 complemented yeast are resistant with only marginal exclusion being evident with 3 μl applied. As expected the AGD61-27 mutant (which lacks Lcb1 and IPC synthase) is resistant to both drugs thereby confirming their target specificity.

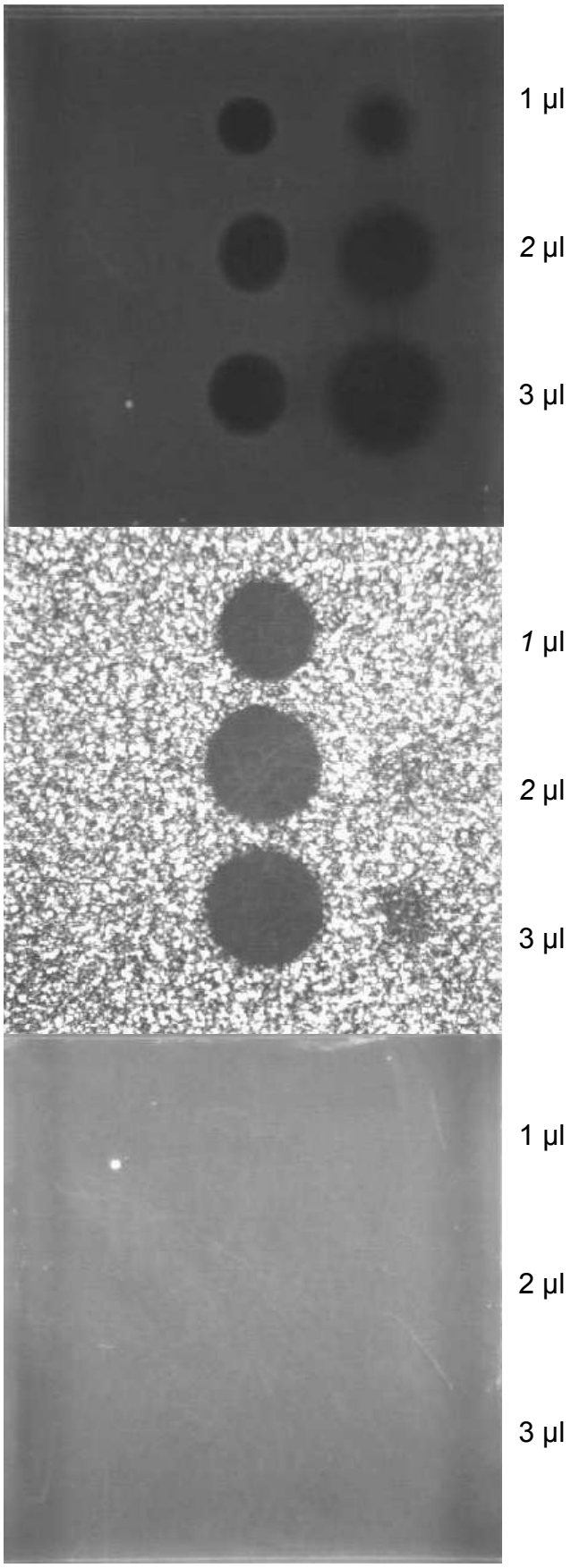

Figure 4.4.1 is EPI white images of yeast embedded YPD plates. Top and middle are transformed ScLCB2 deficient yeast. Bottom is the AGD61-27 strain. Top has been transfected with wild type ScLCB2 on the pRS426 plasmid. It shows the strain is successfully complemented by the negative control whilst Ab A and Myriocin both inhibit yeast growth. Middle is ScLCB2 deficient mutants complemented with TgSPT1 from *Toxoplasma gondii*. Again it successfully returns SPT activity and rescues parasites whilst retaining susceptibility to Ab A. The enzyme is only susceptible to Myriocin at higher concentration levels. Bottom is the AGD61-27 mutant strain. It is unsurprisingly resistant to all treatments as it does not utilise classical sphingolipid biosynthetic pathways.

4.5.1 Concluding remarks

Clearly TgSPT1 is an unusual SPT which is relatively resistant to myriocin when compared to the yeast enzyme. As discussed in **Chapter 3** two SPT orthologues are encoded in the *T.gondii* genome. However, they demonstrate 91% similarity and 82% identity in predicted amino acid sequence and both harbor a PLP binding site, therefore they are unlikely to be homologues of the eukaryotic Lcb1 and Lcb2. This lack of homology to conventional heterodimeric eukaryotic SPT may explain the resistance of TgSPT1 to myriocin. Overall the apicomplexan enzymes appear to be more closely related to the prokaryotic SPTs found in sphingomonads such as *Sphingomonas paucimobilis* and *Sphingobacterium multivorum* in which a single gene encodes a homodimeric SPT (Yard *et al*, 2007; Ikushiro *et al,* 2007). However the effect of myriocin is unstudied in the sphingomonad bacteria and warrants further investigation. This could be conducted as above if bacterial SPT was shown to complement the auxotrophic yeast strain.

As expected, both the ScLcb2 and TgSPT1 complemented lines are Ab A susceptible as described above. Notably, previous work has shown that *T. gondii* replication is irreversibly inhibited when the cells are exposed to Ab A (Sonda *et al*, 2005). This indicated that the parasite possesses a fungal-like IPC activity. However, whilst unpublished work in the laboratory has identified the *T. gondii* IPC synthase it has also indicated that any growth inhibition is likely to be due to off target effects (Wandsadhipathi-Kannangara and Denny, unpublished).

Complex sphingolipids are rarely found in Eubacteria, which tend to make phosphatidyl glycerol as described in *E.coli* (Chang and Kennedy, 1969; White and Tucker, 1969). The sphingomonads represent a rare group of bacteria which produce ceramides which can stimulate eukaryotic signalling pathways (Minamino *et al*, 2003). However they do lack further proteins such as IPC synthase which *T.gondii* and Fungal species possess. Therefore the sphingolipid

biosynthetic pathway of *T.gondii* and other apicomplexans could be a regarded as a complex ‘mix’ of both prokaryotic (i.e. SPT) and eukaryotic enzymes. This could be hypothesized to be the result of lateral transfer of the bacterial genes from endosymbionts, followed by the subsequent secondary loss of eukaryotic coding sequences. Such a process would have occurred within the *Apicomplexa* after divergence from other eukaryotes.

Chapter 5 Localisation of TgSPT1 in Toxoplasma

5.1.1 Aims

The aim of this experimental work was to determine the subcellular localisation of TgSPT1 using immuno-fluorescent microscopy and to investigate whether the predicted membrane-anchor domain is sufficient for this.

5.2.1 Introduction to cellular localisation in TgSPT1

The cellular localization of serine palmitoyltransferase (SPT) protein in *Toxoplasma gondii* is of great interest given its highly unusual, divergent nature (see **Chapter 3** for details). As described, the *T. gondii* TgSPT1 can constitute enzyme activity alone. In contrast to other eukaryotes, 2 sub-units (Lcb1 and 2) are required for an endoplasmic reticulum (ER) localized activity (Buede *et al*, 1991; Nagiec *et al*, 1994; Mandon *et al*, 1992). However, the apicomplexan enzyme bears more similarity to the bacterial SPT: a soluble, homodimeric protein encoded by a single gene in the sphingomonads (Ikushiro *et al*, 2003; Ikushiro *et al*, 2009).

5.3.1 Localisation of TgSPT1 in Toxoplasma gondii

Transient transfection of *T.gondi* was confirmed using plasmid ptubGRASP-RFP/sagCAT (Pfluger *et al*, 2005; a kind gift from Dr Kristin Hager, University of Notre Dame; data not shown). It is worth noting that the level of expression, although not quantified, showed significant increases with increasing quantities of plasmid DNA up to 50µg per transfection. Finally, maximal expression post transfection is seen after approximately 36 hours (Weiss and Kim, 2007). Subsequently 50µg of plasmid DNA was employed per transfection and images were obtained after 36 hours.

Using homology modeling techniques (HMMtop, Tusnády and Simon, 1998; Tusnády and Simon, 2001) it was predicted that the first 47 amino acids within the TgSPT1 sequence contain the localisation signal, with residues 38-47 forming the sole transmembrane helical domain. These analyses also suggested that amino acids 1-37 are lumenal whilst 48-571 are cytosolic. To confirm the role of this 47 amino acid N-terminal domain, fusion proteins shown in figure 5.3.1 were transiently transfected into *T. gondii* as described in Material and Methods.

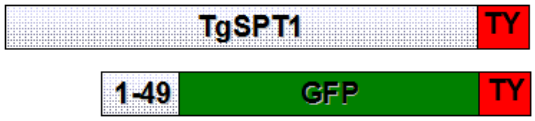

Figure 5.3.1a shows how our original construct with the wild type protein being expressed with a TY tag from the pT8 vector (a kind gift from Prof Dominique Soldati, University of Geneva). The 1-49 truncated form is also shown, with the N-terminal domain fused to GFP and TY within pT8. The monoclonal antibody BB4 (a kind gift from Prof Keith Gull, Oxford) was used to probe for the TY epitope by immuno-fluorescence.

Figure 5.3.1a (top) shows pT8-TgSPT1::TY as constructed by Dr. S. Pratt (Durham) by blunt ended TOPO cloning from *T. gondii* cDNA. Following transfection of this plasmid construct as described, BB4 was used to localize the expressed protein in *T. gondii* by immuno-fluorescence (**Figure 5.3.1b, left**). The reticular expression pattern of TgSPT1-TY indicates that the protein is located in the endoplasmic reticulum of the cell. This is further supported by a basal (the apicoplast is at apical) projection of stained material as previously observed for the *T. gondii* ER (Pfluger *et al*, 2005; **Figure 5.3.1b, right**). This localisation indicated that despite the unusual nature of TgSPT, it is ER localised as in other eukaryotes (Hager *et al*, 1999).

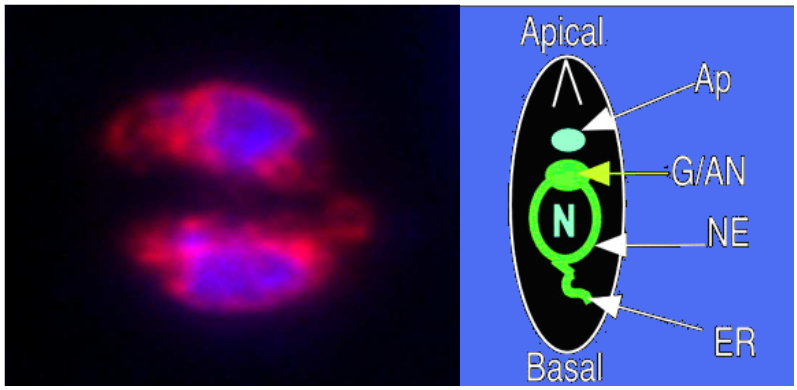

Figure 5.3.1b (left) Fluorescent microscopy image of two *T.gondii* parasites expressing TgSPT::TY (red). The DAPI (blue) stains DNA and shows the nucleus of each as well as 1 apicoplast per cell. Figure 5.3.1b (right) Schematic view of a *Toxoplasma* cell. Note how there is a basal projection of the ER (Adapted from Pfluger *et al*, 2005).

5.4.1 Role of N-terminal domain of TgSPT1 in localisation

This experiment was performed to see if the N-terminal 49 amino acids of TgSPT1 were sufficient to localise a fusion protein to the ER. This would give us new insight into protein targeting in *T. gondii*, which is in its infancy.

To achieve this the first 49 amino acids of TgSPT1 were cloned into the pT8-GFP-TY vector to give pT8-TgSPT1-49::GFP::TY, the fusion depicted in **5.3.1a (lower)**. The GFP does not fluoresce effectively within the cell due to the cleavage, therefore the TY tag was probed using the BB4 monoclonal antibody as above. As mentioned the N-terminal contains the predicted transmembrane domain.

Figure 5.4.1 shows two transfected *T. gondii* cells expressing TgSPT1-49::GFP::TY. Although the localisation was more diffuse than that seen with the full-length protein, the fusion is clearly targeted to subcellular bodies. Therefore the N-terminal 49 amino acids of TgSPT1 appear sufficient to target a fusion protein to membranous compartments. This is in agreement with homology modeling which predicts it contains a membrane-spanning domain. However further work is required establish precisely where it directs the fusion protein.

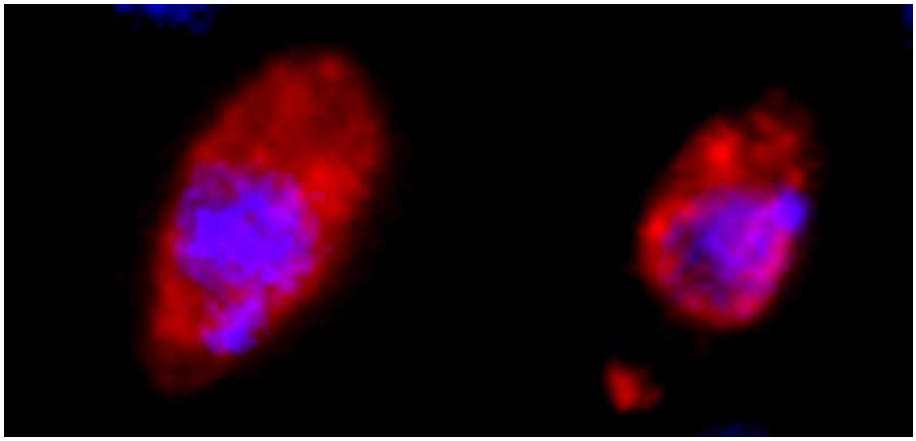

Figure 5.4.1 Fluorescent microscopy image of two *T.* gondii parasites expressing TgSPT1-49::GFP::TY fusion protein (red). The DAPI stained (blue) nucleus and the apicoplast is visible in each.

Notably, removing this 49 amino acid leader sequence prevented the detectable expression of TgSPT1 in *T. gondii* (Pratt and Denny, unpublished). This implies that if the enzyme is free in the cytosol then toxicity results. Adding a short myc amino acid tag to the N-terminus of TgSPT1 had the same affect (Pratt and Denny, unpublished) indicating that the 28 amino acid residues, which precede the transmembrane domain, are important for targeting the protein correctly.

5.5.1 Future research

Figures 5.3.1b (left) and **5.4.1** show localisation by implication but do not provide a definitive answer. In order to fully establish the localisation of TgSPT1 and TgSPT1-14::GFP::TY further analyses are necessary. In the first instance co-staining for known *T. gondii* ER markers would allow definitive localization of the protein as being in the ER. This has been attempted by transient co-expression of P30::GFP::HDEL (a kind gift from Dr Kristin Hager, University of Notre Dame; Hager et al, 1999) and TgSPY::TY. However, expression of the HDEL ER marker was unsuccessful in our hands (Bruce and Denny, unpublished). Ultrastructural analyses employing immuno-electron microscopy or stimulated emission depletion microscopy could also definitively localize the proteins (Hell and Kroug, 1995). Previously the Lcb subunit was located in the ER through highly purified mouse liver samples (Mandon *et al*, 1992). Therefore

an alternative to fluorescence microscopy could involve a similar method, the separation of subcellular fractions, although this has yet to be established for isolation of the ER and Golgi apparatus from *T. gondii*.

Chapter 6 Observing Endocytosis in *Toxoplasma gondii*

6.1.1 Aims of the endocytosis experiments

Using FITC-labeled dextran, Texas red-labeled concanavalin A and BODIPY-sphingomyelin with isolated extracellular *T.gondii* tachyzoites their endocytic potential of was studied. These molecules were fluorescently tagged and their uptake was examined by fluorescence microscopy and quantified by a fluorescence plate reader.

6.2.1 Introduction to T. gondii Endocytosis

FITC-labelled dextran was used to measure fluid phase (FP) endocytosis, whilst concanavalin A (conA) was utilised as a marker of receptor mediated (RM) endocytosis (Botero-Kleiven *et al*, 2001; Eshafani *et al*, 1986). Fluid phase endocytosis results from invaginations of the plasma membrane which 'pinch' off to form vesicles, and in doing so trap macromolecules within the extracellular matrix of the vesicle. Dextran enters the eukaryotic cells via FP endocytosis and, as a well characterised marker for this process, has been employed in this study (Falcone *et al*, 2006; Fernandez *et al*, 2006). Concanavalin A (conA) is a lectin which stimulates agglutination with glycoproteins within the plasma membrane. In doing this it stimulates signalling cascades and ultimately internalization (Pagano *et al,* 1991). It was for this reason it will be utilised here as a marker of RM-endocytosis.

In *T. gondii* intracellular tachyzoites have been shown to acquire cholesterol intercepted from the host cell LDL-degradative pathway and bradyzoites have been shown to pick up morsels of cyst matrix through their micropore (Coppens *et al*, 2000; Nichols *et al*, 1994). However, little is known about the endocytic activity of extracellular parasites, although extracellular tachyzoites have been observed to slowly internalize the FP endocytic tracer horseradish peroxidise

through vacuole formation at the base of this structure (Nichols *et al*, 1994). The uptake of dextran (at low levels) by extracellular tachyzoites has also been reported (Botero-Kleiven *et al*, 2001). In eukaroyotes glycans and glycoconjugates are taken up by RM-endocytosis. Based on this it was hypothesised that host cell glycosaminoglycan could be endocytosed by *T. gondii* during invasion when the glycan groups would come into contact with the parasite (Varki, 1999). Indeed endocytosis of the FITC-labelled glycosaminoglycan, heparin, has been measured in *T. gondii* indicating the occurrence of RM-endocytosis (Botero-Kleiven *et al*, 2001).

Despite this evidence for both FP and RM-endocytosis *T. gondii* the published studies are few in number and limited.

Sphingomyelin is a complex phosphosphingolipid found predominantly in animal cells. There is evidence that intracellular *T.gondii* tachyzoites can actively scavenge cholesterol (associated with sphingolipids in lipid rafts) and glycosphingolipid gangliosides (de Melo and de Souza, 1996).

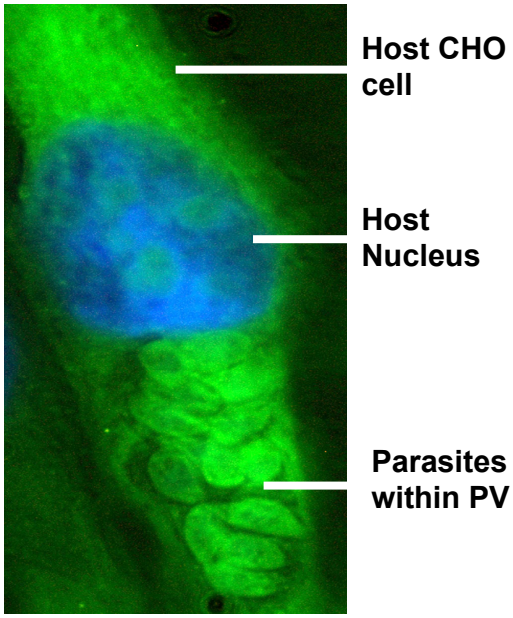

Figure 6.2.1 Fluorescent microscopy of a CHO cell infected with RHΔHX tachyzoites. Fluorescent BODIPY-sphingomyelin is clearly taken up by the host cell and parasites within the parasitophorous vacuole. This is very rapid, less than 1 minute incubation after loading plasma membrane of host with BODIPY-sphingomyelin (Pratt and Denny, unpublished).

Figure 6.2.1 shows the uptake of fluorescent-labeled BODIPY-sphingomyelin by the host cell and intracellular parasites following a short 37°C incubation (Pratt and Denny, unpublished). The parasitophorous vacuole membrane (PVM) functions as a molecular sieve allowing molecules of up to 1926 Da to diffuse through it (Schwab *et al*, 1994). Therefore, molecules such as Lucifer yellow (474 Da) can pass through the PVM readily and enter the PV in a passive manner. However, sphingomyelin (molecular weight approximately 35000 Da) would be unable to passively diffuse through the PVM and the uptake observed represents a true reflection of active *in situ* sphingomyelin scavenging. To fully investigate whether sphingomyelin is endocytosed by *T. gondii* it was necessary to develop a straight-forward assay involving extracellular tachyzoites. Therefore, freshly released parasites were assayed for uptake of the FP marker dextran, the RM marker con A as well as sphingomyelin. The viability of the freshly isolated *T. gondii* was verified using trypan blue exclusion assay (Tennant, 1964). Greater than 99% of cells demonstrated signs of viability.

6.3.1 Dextran endocytosis assay in extracellular parasites

Dextran is a complex, branched polysaccharide made of many glucose molecules. It is a large molecule (in our experiments 10,000 Da**),** far too large to passively diffuse through the plasma membrane of the host or the PVM. By incubating freshly isolated *T.gondii* with dextran FP-endocyotosis was measured. Chilling and labeling cells on ice blocks active endocytosis, therefore comparing these with parasites labeled at 37°C allowed quantification of FP endocytosis. **Figure 6.3.1** clearly shows there is very little difference in fluorescence between the two data sets. This indicates that, under these conditions, FP endocytosis does not occur in extracellular tachyzoites. For reasons unknown this finding conflicts with that of (Botero-Kleiven *et al* 2001).

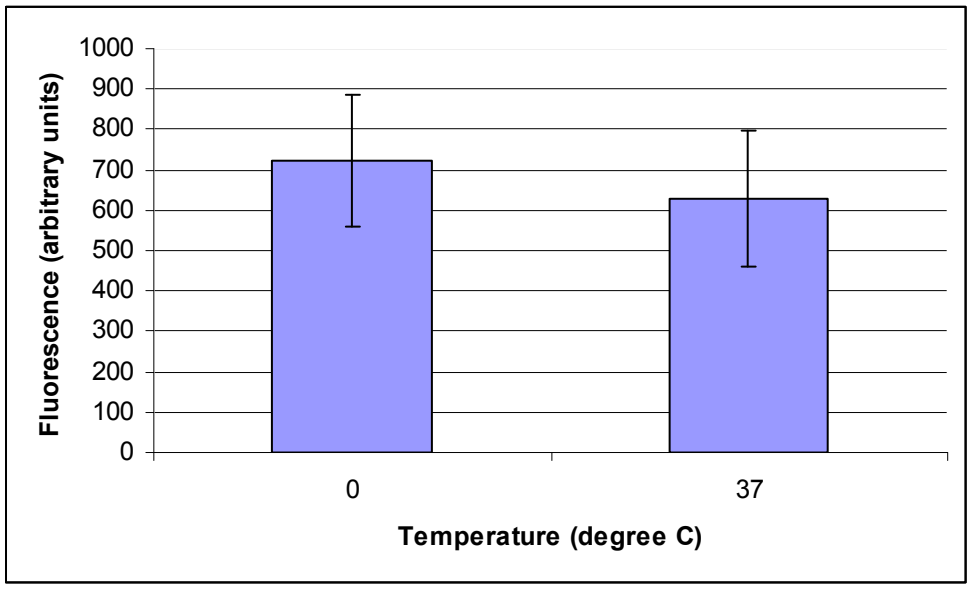

Figure 6.3.1 Quantified uptake of fluorescent dextran at 0 °C and at 37 °C Cells were incubated in FITC-labeled dextran for 2 hours and analysed using a fluorescence plate reader. Samples were measured in triplicate format.

6.4.1 Concanavalin A endocytosis in Toxoplasma gondii

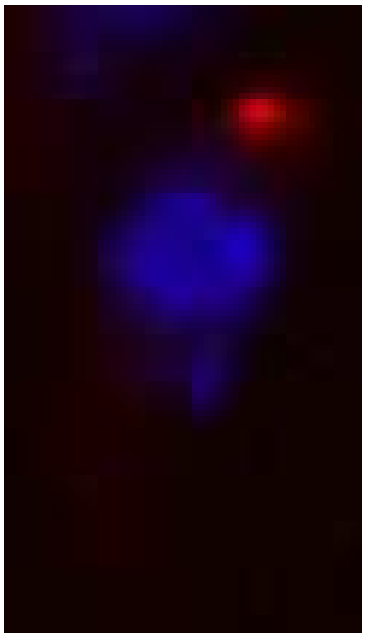

Figure 6.4.1 *T.gondii* labeled with Texas Red concanavalin A (courtesy of Dr. P. Denny). There is clear localisation of con A (red) at the basal end of the cell. The DNA is DAPI-stained (blue), the smaller body is the apicoplast, which is orientated towards the apical end of the tachyzoite.

In mammalian cells con A agglutination of glycoproteins stimulates RM-endocytosis (Pagano *et al*, 1991). However, **Figure 6.4.1** clearly shows that whilst there is aggregation of macromolecules of the basal end of the parasite, they are not internalized. This suggests that the extracellular parasite does not undertake RM-endocytosis, at least of con A. As mentioned above, previous studies have suggested that such isolated tachyzoites take up heparin by RM-endocytosis (Botero-Kleiven *et al* 2001). Unfortunately, the opportunity to replicate these analyses was not available.

6.5.1 Sphingomyelin endocytosis assay in T. gondii

Sphingomyelin is a complex sphingolipid produced in nearly all animal cells. They are important membrane constituents and powerful signaling molecules. **Figure 6.5.1** shows quantified fluorescence in samples labeled with BODIPY-sphingomyelin on ice (no endocytosis) before incubation at 37°C for the defined

time period and subsequent back-extraction with fat-depleted BSA to remove plasma membrane associated label. At time point 0, fluorescent is clear even after back-extraction with BSA. If endocytosis occurs then fluorescent signal should increase over time as the BODIPY-sphingomyelin enters the cells and is protected from extraction with BSA. However, there is a general decline in total fluorescence to an asymptote. This was unexpected as even in the absence of endocytosis no significant change in the levels of fluorescence would be predicted.

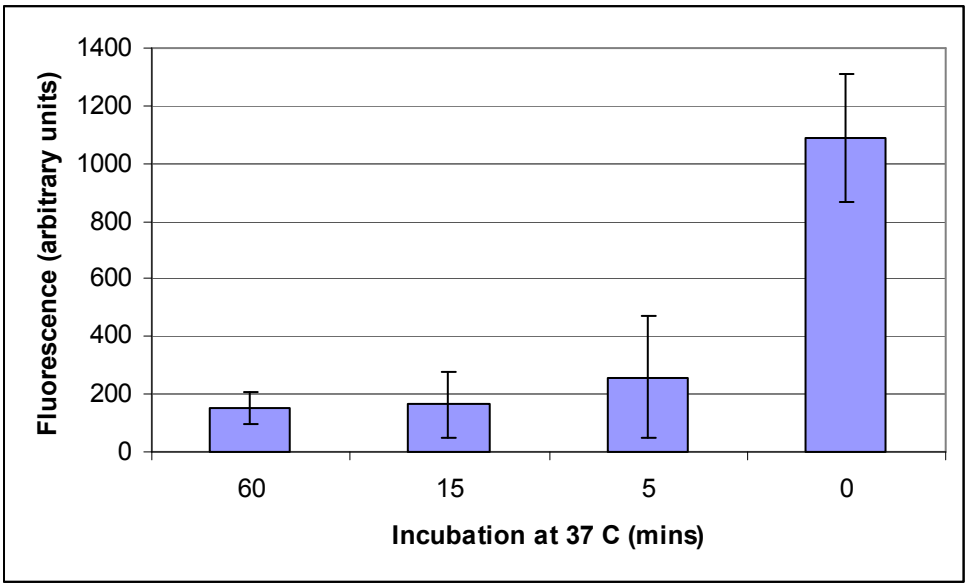

Figure 6.5.1 Quantified uptake of fluorescent dextran 37 °C for the time indicated, perform by a fluorescence microplate reader. Samples were measured in triplicate.

Visualization using fluorescence microscopy was performed on the samples and the presence of BODIPY-sphingomyelin was clear only at time point 0.

6.6.1 Future work following on from these results

The data collected indicates that extracellular parasites are relatively inert with respect to FP and RM-endocytosis, and the uptake of sphingomyelin. However, 22000 Da cholesterol is actively scavenged by intracellular parasites (Coppens *et*

al, 2000), as is sphingomyelin (**figure 6.2.1**) Future studies should investigate whether host cell endocytosed con A and dextran is trafficked to the PV.

Of course it could be that the markers chosen to analyse these processes (e.g. dextran and conA) are inappropriate. However, dextran endocytosis has been demonstrated in the fellow apicomplexan *Plamodium falciparum* when isolated form its host red blood cell. In addition, ablation of dynamin caused arrest of parasite growth and a decrease in haemoglobin uptake. This maybe attributed to the fact that dynamin is required for the 'pinch off' off endocytic vesicles and therefore endocytosis (Zhou *et al*, 2009). Notably, dyanmin is also involved in the formation of clatharin-coated pits (involved in RM-endocytosis) and their detachment from the plasma membrane in *P.falciparum* (Nankoe and Sever, 2006). Therefore inhibition of dynamin could block both FP and RM endocytosis. The creation of *T.gondii* dynamin mutants would, in the future, allow further investigation of the role of endocytosis in parasite pathogenicity.

Chapter 7 Conclusions and Future Work

7.1.1 Bioinformatic analyses of the predicted apicomplexan *SPT*

It was established that there is primary sequence conservation between sphingomonad SPTs, apicomplexan SPTs and the eukaryotic SPT subunit 2 (Lcb2). Within the catalytic domain of these functional proteins there is absolute conservation of key amino acids (such as the lysine and the DXAH motif). Upon the construction and analyses of neighbour-joining, maximum likelihood phylogenetic trees there is tentative support for the apicomplexan SPT being more closely related to the prokaryotic enzymes than either eukaryotic Lcb1 or Lcb2. *Toxoplasma* is unique amongst the *Apicomplexa* in having two SPT genes, However given the very high sequence identity they are most likely to be a result of a gene duplication event and not a Lcb1 and 2 heterodimer model. The origin of the apicomplexan SPT can only be speculated at, although lateral transfer from a bacterial endosymbiont remains a possibility. Notably, whilst *Toxoplasma gondii* (and other apicomplexans) encode a prokaryotic-like SPT it also possesses eukaryotic-like sphingolipid biosynthetic enzymes (e.g. IPC synthase). Therefore the sphingolipid biosynthetic pathway of *T. gondii* may be a chimera of the eukaryotic and prokaryotic-like enzymes.

7.1.2 Complementation of ScLcb2 deficient yeast

It was demonstrated that TgSPT1 can rescue ScLcb2 deficient yeast clearly supporting its initial identification. Furthermore, it was shown that TgSPT1 is resistant to the well characterised SPT inhibitor myriocin. This resistance supports the idea of their being a relatively high evolutionary divergence of TgSPT1 from the eukaryotic orthologues.

7.1.3 Subcellular localisation of TgSPT1 and a truncated fusion protein

Expressing Ty-tagged TgSPT1 and TgSPT1-49::GFP proteins in *T. gondii* tachyzoites facilitated subcellular localisation by immuno-fluorescence. TgSPT was found in reticular structures surrounding the nucleus with the characteristic basal projection associated with ER localised proteins. TgSPT1-49::GFP showed a more diffuse expression pattern, although it was clear that the 49 amino acid N-terminal domain of TgSPT was sufficient to target the protein to membranous domains. Notably, removing this domain negated expression (Pratt and Denny, unpublished) suggesting a misfolded protein response and degradation by the proteosome (Zhang and Kaufman, 2006).

7.1.4 Endocytosis assays on extracellular tachyzoites

The fluid phase and receptor-mediated endocytic activity of extracellular tachyzoites was measured with FITC-Dextran (10000Da), Texas red labeled concanavalin A and BODIPY-sphingomyelin. There was no active uptake of these markers, suggesting that the extracellular parasite is relatively inert in endocytic terms compared with intracellular *T. gondii*. Alternatively, it is possible that the markers used in our study were not appropriate. Obviously this requires further investigation.

7.2.1 Bioinformatic analyses of other enzymes involved in apicomplexan *sphingolipid biosynthesis*

In light of the apparent prokaryotic origin of the apicomplexan SPT, other enzymes of the protozoan sphingolipid biosynthetic pathway should be analysed to further understand its evolutionary origin. The enzyme IPC synthase has already been isolated from *T. gondii* (Wansadhipathi-Kannangara and Denny, unpublished) and evolutionary analyses of this and other key proteins would facilitate a clearer understanding of the origin of apicomplexan sphingolipid biosynthesis.

7.2.2 Comparative analyses of TgSPT with bacterial SPT

The ability of the soluble bacterial SPT to complement ScLcb2 deficient yeast should be analysed to begin to establish whether the subcellular localisation of the enzyme is key to eukaryotic function. In addition, given the resistance of TgSPT to myriocin, it would be of interest to establish if the bacterial enzyme is susceptible. This could be achieved following expression in ScLcb2 deficient yeast. The results may add to our understanding of enzyme function and evolution.

7.2.3 Further localisation of TgSPT

Given the reticular expression pattern of the TgSPT1 in *T. gondii* it was proposed that it was, like other eukaryotic SPTs, ER localised. Confirmation of this has been attempted by co-localisation with the ER marker P30::GFP::HDEL. However this has so far proven unsuccessful (Bruce and Denny, unpublished) and the use of alternative markers will have to be investigated. Alternatively, higher resolution immuno-electron microscopy may confirm the localization, as could subcellular fractionation of the parasite.

7.2.4 Endocytosis assays in intracellular tachyzoites

Given our results it seems likely that extracellular tachyzoites are inert with respect to fluid phase and receptor-mediated endocytosis. In contrast, the uptake of both cholesterol and sphingomyelin has been by intracellular parasites has been observed (Pratt and Denny, unpublished; Coppens *et al*, 2000). Notably, dynamin is essential for the 'pinching-off' of endocytic vesicles from the plasma membrane. Therefore, dynamin defective *T. gondii* mutants could be used to investigate the role of clathrin-mediated endocytosis in the scavenging of host lipids. Such studies will be important to more fully understand the intimate relationship between the parasite and its host.

Chapter 8 - Appendices

8.1 List of genes and accession numbers

Opisthokonta			
Metazoa			
HsLCB1	Homo sapiens	EAW62806	www.ncbi.nlm.nih.gov
HsLCB2	Homo sapiens	EAWO15270	www.ncbi.nlm.nih.gov
MmLCB1	Mus musculus	NP_033295.2|	www.ncbi.nlm.nih.gov
MmLCB2	Mus musculus	NP_035609.1	www.ncbi.nlm.nih.gov
DmLCB1	Drosophila melanogaster	DM_7303358	www.ncbi.nlm.nih.gov
DmLCB2	Drosophila melanogaster	DM_7298232	www.ncbi.nlm.nih.gov
CeLCB1	Caenorhabditis elegans	WP:CE08323	www.ncbi.nlm.nih.gov
CeLCB2	Caenorhabditis elegans	WP:CE27380	www.ncbi.nlm.nih.gov
DrLCB1	Danio rerio	NP_001018307	www.ncbi.nlm.nih.gov
DrLCB2	Danio rerio	NP_001108213	www.ncbi.nlm.nih.gov
NvLCB1	Nematostella vectensis	E_gw.173.15.1	http://genome.jgi-psf.org/
NvLCB2	Nematostella vectensis	pg.C_520020	http://genome.jgi-psf.org/
MbLCB1	Monosiga brevicollis	pg.C_520020	http://genome.jgi-psf.org/
MbLCB2	Monosiga brevicollis	pg.C_360003	http://genome.jgi-psf.org/
Fungi			
ScLCB1	Saccharomyces cerevisiae	YMR296C	www.old.genedb.org
ScLCB2	Saccharomyces cerevisiae	YDR062W	www.old.genedb.org
CnLCB1	Cryptococcus neoformans	Q5KNA1_CRYNE	www.uniprot.org/
CnLCB2	Cryptococcus neoformans	CNAG_01477.2	www.uniprot.org/
SpLCB1	Schizosaccharomyces pombe	NP_595848	www.old.genedb.org
SpLCB2	Schizosaccharomyces pombe	XP_001713103	www.old.genedb.org
AnLCB1	Aspergillus nidulans	ANID_03728	www.uniprot.org/
AnLCB2	Aspergillus nidulans	ANID_01102	www.uniprot.org/
RoLCB1	Rhizopus oryzae	RO3G_05590	www.uniprot.org/
RoLCB2	Rhizopus oryzae	RO3G_05590	www.uniprot.org/
Archaeplastida			
PtLCB1	Populus trichocarpa	pg.C_LG_XII000006	http://genome.jgi-psf.org/
PtLCB2	Populus trichocarpa	pm.C_LG_XII0379	http://genome.jgi-psf.org/

Chromalveolata			
Apicomplexa			
TgSPT1	Toxoplasma gondii	TGGT1_032140	www.toxodb.org
TgSPT2	Toxoplasma gondii	TGME49_090970	www.toxodb.org
PfSPT	Plasmodium falciparum	PF14_0155	www.genedb.org
PvSPT	Plasmodium vivax	PVX_085665	www.genedb.org
EtSPT	Eimeria tenella	SNAP00000006674	www.old.genedb.org
CmSPT	Cryptosporidium muris	B6ACS8_CRYMR	www.genedb.org
Excavata			
Discicristata			
TbLCB1	Trypanosoma brucei	Tb927.4.1020	http://genome.jgi-psf.org/
TbLCB2	Trypanosoma brucei	Tb10.70.3220	http://genome.jgi-psf.org/
LmLCB1	Leishmania major	LmjF34.3740	www.genedb.org
LmLCB2	Leishmania major	LmjF35.0320	www.genedb.org
Proteobacteria			
SpSPT	Sphingomonas paucimobilis	Q93UV0_PSEPA	www.uniprot.org/
SmSPT	Sphingobacterium multivorum	A7BFV6_9SPHI	www.uniprot.org/

Chapter 9 References

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